A STUDY ON IRON STATUS & THYROID FUNCTION : ? MUTUAL RELATIONSHIP IN HYPOTHYROIDISM

Dissertation submitted for

M.D. BIOCHEMISTRY

BRANCH – XIII DEGREE EXAMINATION

THE TAMILNADU DR.M.G.R.MEDICAL UNIVERSITY

CHENNAI – 600 032

TAMILNADU

MAY 2019

BONAFIDE CERTIFICATE

This is to certify that this dissertation work entitled "**A STUDY ON IRON STATUS & THYROID FUNCTION : ? MUTUAL RELATIONSHIP IN HYPOTHYROIDISM"** is the original bonafide work done by **Dr. K.S.GOKILAVENI**, Post Graduate Student, Institute of Biochemistry, Madras Medical College, Chennai under our direct supervision and guidance.

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DECLARATION

I, Dr. K.S.GOKILAVENI, Post Graduate , Institute of Biochemistry, Madras Medical College, solemnly declare that the dissertation titled "**A STUDY ON IRON STATUS & THYROID FUNCTION : ? MUTUAL RELATIONSHIP IN HYPOTHYROIDISM"** is the bonafide work done by me at Institute of Biochemistry, Madras Medical College under the expert guidance and supervision of our Director and **Prof. Dr. K.RAMADEVI M.D,.Ph.D**.,, Institute of Biochemistry, Madras Medical College. The dissertation is submitted to the Tamil Nadu Dr. M.G.R Medical University towards partial fulfillment of requirement for the award of M.D., Degree (Branch XIII) in Biochemistry.

Place: Chennai

Date: **Dr.K.S.GOKILAVENI**

SPECIAL ACKNOWLEDGEMENT

The author gratefully acknowledges and sincerely thanks **Dr .R. Jayanthi, MD., FRCP (Glasg),** Dean, Madras Medical College and Rajiv Gandhi Govt. General Hospital, Chennai, for granting her permission to utilize the facilities of this Institution for the study.

ACKNOWLEDGEMENT

The author expresses her warmest respects and profound gratitude to **Dr. K. Ramadevi, M.D,.Ph.D**.,, Director and Professor, Institute of Biochemistry, Madras Medical College, Chennai, for her able guidance, constant encouragement, support and valuable time but for which this dissertation could not have been made possible.

The author expresses her heartfelt gratitude to her co -guide **Dr. A. Veena Juliette** Assistant Professor, Institute of Biochemistry, Madras Medical College, Chennai, for her constant and valuable guidance, unfailing support, encouragement and inspiration throughout the period of her study.

The author in particular, is extremely thankful to **Dr. Sripriya M.D**, Institute of General Medicine & **Professor Dr.Tito,** Head of the department, Department of Endocrine Surgery, Rajiv Gandhi Government General Hospital, Chennai, for granting permission to obtain blood samples from the patients.

The author expresses her sincere gratitude to the **Professors Dr.R.Chitraa, Dr.K.Pramila, Dr.Sumathy.S & Dr. Chelladurai,** Institute of biochemistry, Madras Medical College, for their guidance and support.

The author expresses her warm respects and sincere thanks to **Dr.V.Ananthan and Dr. K.R.Minu Meenakshi Devi,** Assistant Professor, Institute of biochemistry, Madras Medical College for their guidance regarding the practical issues of research which is beyond the scope of textbooks.

The author expresses her respects and sincere thanks to all other **Assistant Professors** Institute of Biochemistry, Madras Medical College, for their guidance and support.

The author expresses warm respects to the members of the Institutional Ethics committee for approving the study. The author is indebted to the patients and persons from whom blood samples were collected for conducting the study. The author expresses her special thanks to Biochemistry Laboratory Staff, for their timely help and cooperation during sample collection.

The author gratefully acknowledges the help rendered by **Dr.S.Boobala Aravinthan M.D.,** for the statistical analysis of the study.

The author expresses her special thanks to her mother **Mrs.A.Parameswari** and her husband **Dr.S. Boobala Aravinthan** and her collegue **Dr.M.Dilagamari** and other family members for the moral support and encouragement extended by them.

CONTENTS

ABBREVIATIONS

1.	Apo	-	apolipoprotein
2.	BMR	-	Basal metabolic rate
3.	CBC	-	Complete blood count
4.	Cp	-	Ceruloplasmin
5.	DIT	-	Diiodothyronine
6.	DMT	-	Divalent metal transporter
7.	ELISA	-	Enzyme linked immune sorbent assay
8.	EDTA	-	Ethyelene diamine tetra acetic acid
9.	ER	-	Endoplasmic reticulum
10.	FeOOH	-	Ferric oxyhydroxide
11.	FSH	-	Follicle stimulating hormone
12.	HCG	-	Human chorionic gonadotropin
13.	HCP	-	Heme carrier protein
14.	Hb	-	Hemoglobin
15.	HH	-	Hereditary Hemochromatosis
16.	HRP	-	Horse radish peroxidase
17.	HMP	-	Hexose monophosphate
18.	IDA	-	Iron deficiency anemia
19.	IRE	-	Iron regulatory element
20.	IRP	-	Iron regulatory protein
21.	kDa	-	Kilo Dalton
22.	LH	-	Luetinising hormone
23.	MIT	-	Mono iodo thyronine
24.	NADPH	-	Nicotinamide adenine dinucleotide phosphate
25.	Na-K-ATPase	-	Sodium potassium ATPase

26.	PAX-8	-	Paired box gene 8
27.	PTH	-	Parathormone
28.	RNA	-	Ribonucleic acid
29.	RBC	-	Red blood cells
30.	sTfR	-	Soluble transferrin receptor protein
31.	TPO	-	Thyroid peroxidase
32.	TBG	-	Thyroid binding globulin
33.	T3	-	triiodothyronine
34.	T4	-	Tetraiodothyronine
35.	fT3	-	free triiodothyronine
36.	fT4	-	free Tetraiodothyronine
37.	TR	-	Thyroid receptor
38.	TRH	-	Thyrotropin releasing hormone
39.	TSH	-	Thyroid stimulating hormone
40.	TfR	-	transferrin receptor
41.	Tf	-	Transferrin
42.	TIBC	-	Total iron binding capacity
43.	TMB	-	Tetramethylbenzidine
44.	UTR	-	untranslated region
45.	WHO	-	World health organization

Introduction

INTRODUCTION

Thyroid diseases are common worldwide. In India also we have significant burden of thyroid diseases among which hypothyroidism is common that can be easily diagnosed and is a treatable condition. It often presents with non specific symptoms and hence diagnosed by measuring the thyroid hormone levels in blood[(1)]. Hypothyroidism when left untreated leads to myxedema, cardiac failure, coma and death.

Thyroid hormones are very important to regulate the metabolism of human body. Hypothyroidism is a clinical entity that develops due to low level of circulating thyroid hormones. Normal thyroid hormone levels depend on many trace elements such as iron, iodine, selenium and zinc both for the synthesis and metabolism of thyroid hormones[(1)]. Deficiencies of these elements can impair thyroid functions [(2)].

Among the micronutrients, iron is important for various functions of our human body. It is essential for cellular growth and differentiation, oxygen binding, transport & storage, enzymatic reactions, immune function, cognitive functions such as mental as well as physical growth. So deficiency of iron by both physiological and pathological means affects mental and physical growth resulting in decreased learning capacity and work productivity[(3)].

Iron deficiency anemia is also a global health problem . WHO estimates that around 2 billion people suffer from anemia among which 50% are due to iron

deficiency[4]. In India prevalence of IDA (iron deficiency anemia) is around 56% (64 million girls) [5].

Iron deficiency is said to manifest due to the depletion of body's iron stores resulting in deficient iron supply to the tissues which need iron for many of its intracellular pathways .

Iron is very intricately related to the thyroid metabolism[6]. Many human and animal studies shows that iron deficiency impairs thyroid metabolism[2]. Iron deficiency has been shown to affect the levels of thyroxine and triiodothyronine and peripheral conversion of T4 to T3 is also affected significantly[1].

Thyroid peroxidase enzyme is the key enzyme which catalyzes almost the entire thyroid metabolic pathway. Iron is an essential cofactor needed for the enzyme thyroperoxidase to function effectively[7]. In status of iron deficiency TPO cannot perform its function of synthesizing thyroid hormones. Hence iron deficiency may be the key factor responsible for hypothyroidism. Martinez – Torrez and coworkers observed that a significant reduction of thyroid hormones, approximately 10 % lower levels of T3 in case of patients with moderate to severe anemia[2]. Actually this aspect is very important while treating hypothyroidism. Because patients with iron deficiency anemia experiences symptoms of sympathetic stimulation such as anxiety , palpitations, arrhythmia etc.,. When these patients have concomitant hypothyroidism the symptoms may worsen on treating them with thyroxine[6].

On treating with thyroxine, levels of erythropoietin improves , enhancing erythropoiesis. This leads to further increased demand for iron stores ,aggravating iron deficiency[6]. Also in hypothyroidism, lack of stimulation of erythroid colony development resulting in decreased level of erythropoietin will lead to the development of anemia and thus affecting iron metabolism.

This leads to a viscious cycle where iron deficiency may be a cause for hypothyroidism or hypothyroidism may cause iron deficiency anemia [6].

There are other mechanisms also by which IDA causes hypothyroidism. Studies shows that the IDA may also lower the levels of 5'deiodinase enzyme which is involved in the peripheral conversion of T4 to T3[2]. There arises a question for the existence of a mutual relationship between hypothyroidism and iron deficiency anemia

There are a panel of tests for measuring blood iron levels which includes the measurement of serum iron levels, TIBC (Total iron binding capacity) levels, ferritin, transferrin saturation levels. Since estimation of iron using the above sets of parameters has a disadvantage of being affected in chronic inflammatory conditions , measurement of iron can be done with the help of combination of new biomarkers such as soluble transferrin receptor protein levels(sTfR), ferritin , sTfR/log ferritin levels[8].

The aim of this present study is to diagnose iron deficiency anemia using hemoglobin levels, peripheral smear, ferritin levels, sTfR levels, sTfR /log ferritin levels among patients with hypothyroidism and to identify if there is a mutual relationship between hypothyroidism and iron deficiency anemia.

Review of Literature

REVIEW OF LITERATURE

THE THYROID GLAND

The thyroid gland is one of the important endocrine glands of the body. Main function of thyroid is to regulate the metabolism at tissue level through thyroid hormones[9].

EMBRYOLOGY OF THYROID GLAND[10]

Thyroid gland develops from the floor of pharynx . The development starts from 24th day of intrauterine life. In due course, the thyroid descends in front of the hyoid bone and laryngeal cartilages. Finally it reaches in front of trachea in the seventh week which has now acquired a small median isthmus and two lateral lobes. By the end of third month at which time the thyroid gland begins to function, the first follicle containing colloid becomes visible. Follicular cells has the colloid which acts as the source for thyroxine hormones.

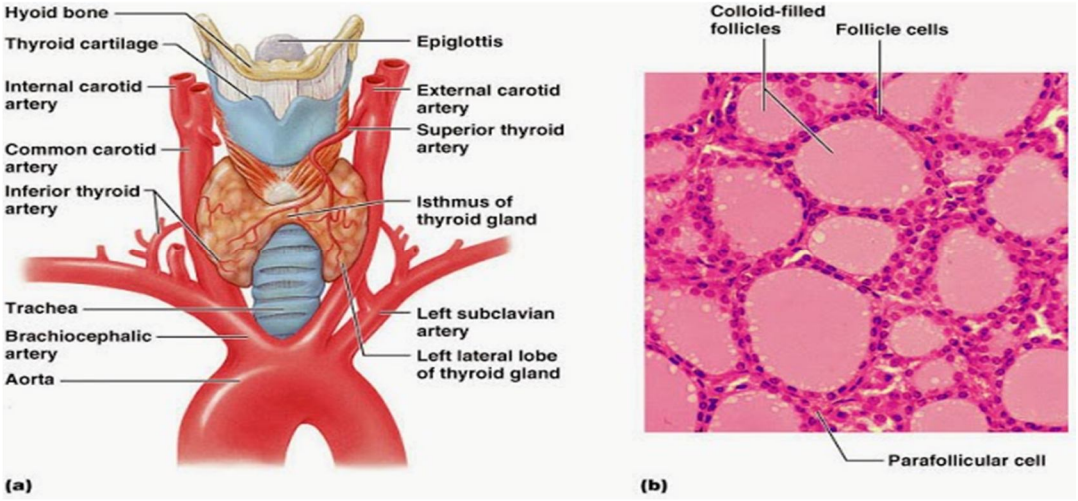
Thyroid hormone raises gradually reaching a plateau by 35 – 37 weeks. T4 and TSH concentrations by 37 weeks will be 10µg/dL and 7 – 10 mIU/L respectively. T3 concentration begins to increase by 30 weeks due to maturation of type I deiodinase activity. At full term of pregnancy T3 concentration reaches 50ng/dL. Soon after birth, TSH, T4 & T3 rapidly increases due to cold stress. TSH reaches a maximum of 70 – 100 mIU/L and falls by 2 to 3 days postpartum to values less than 20 mIU/L.T4 reaches peak concentration of about 17µg/dL and reaches adult concentration by 1 to 2 months of age[11].

Both fetal thyroid stimulating hormone and thyroxine are needed for the normal intrauterine development of central nervous system and skeleton[9].

ANATOMY OF THYROID GLAND[11]

The thyroid gland is a butterfly shaped structure with two lobes. The two lobes are connected by a bridge of tissue called isthmus. The thyroid gland weighs approximately 25 g. It has a rich source of blood supply mainly from thyrocervical arteries and innervations by autonomic nervous system.

The region which is concerned with thyroxine production consists of multiple acini called as follicles. Each follicle is surrounded by epithelial cells and are filled by pink proteinaceous material called colloid. The colloid is rich in thyroglobulin which is a glycoprotein. When the gland is inactive, the follicles expands and when it is active, the follicle shrinks, the colloid is actively reabsorbed into the thyrocytes. Special cells called parafollicular cells are present between the follicles which secrete calcitonin.



Pic courtesy: www.google.com-thyroid anatomy images

The expression of the thyroid follicle cells are mainly regulated by three nuclear transcription factors [11] :

- Thyroid transcription factor 1
- Thyroid transcription factor 2
- Paired box gene 8 (PAX- 8)

FORMATION OF THYROID HORMONE[12]

The products secreted by thyroid glands are iodothyronines :

3,5,3',5' tetraiodothyronine : thyroxine T_4

3,5,3' triiodothyronine : T3

3,3',5' Reverse T3 : rT3

The primary hormone secreted by thyroid is thyroxine with lesser amounts of T3 and minimally reverse T3. The thyroid hormones are the iodinated forms of the amino acid tyrosine.

T3 is biologically more active than T4 as it is produced in the peripheral areas from T4 by deiodination. rT3 is not active biologically.

Substrates for thyroid hormone synthesis :

1. Iodine
2. Tyrosine

IODINE

The dietary iodine is converted to iodide and is absorbed from the gut. The normal daily requirement of iodine is 100 to 200µg . Minimum requirement is 25 µg. Sources are drinking water, fish, cereals, vegetables and iodinated salt. It is a very scarce element and so humans have developed a complex mechanism to utilize this element in its most suitable form.

TYROSINE

It is an aromatic semi essential amino acid which can be iodinated to form the thyroid hormones. **Thyroglobulin** forms the main source for the tyrosine residues. **Thyroglobulin** is the precursor of T4 and T3. It is a large iodinated, glycosylated protein with a molecular mass of 660 kDa. It is composed of two large subunits. It contains 115 tyrosine residues, each of which is a potential site of iodination.

STEPS IN THYROID HORMONE SYNTHESIS [12] :

1.IODIDE TRAPPING

Iodide from circulation is transported into the thyrocytes actively against the electrochemical gradient along with two Na+ ions with the help of the Na^+/I^- symporter. Energy is needed for this transport which is provided by the Na^+K^+ATPase pump. The transport mechanism involved is an example for secondary active transporter. Then the iodide should enter the colloid where the synthesis of thyroxine begins. This is achieved with the help of a passive Cl^-/ I^- exchanger known as pendrin. This is said to be “iodide trapping”.

2.OXIDATION OF IODIDE :

Iodide can be oxidized to any of the three forms :

- iodinium ion or
- free hypoiodite or
- both or a free iodine radical.

TPO enzyme uses hydrogen peroxide to oxidize the tyrosine residue to a free tyrosine radical before it starts reacting with the active iodine. The hydrogen peroxide is obtained from NADPH which is generated from the HMP pathway.

This happens at the interface between the colloid and follicle. After getting oxidized to iodine , it goes and attaches with the 3C of tyrosine residues of the thyroglobulin molecule with the help of the TPO enzyme. This process is called as **organification** of iodine.

3.IODINATION

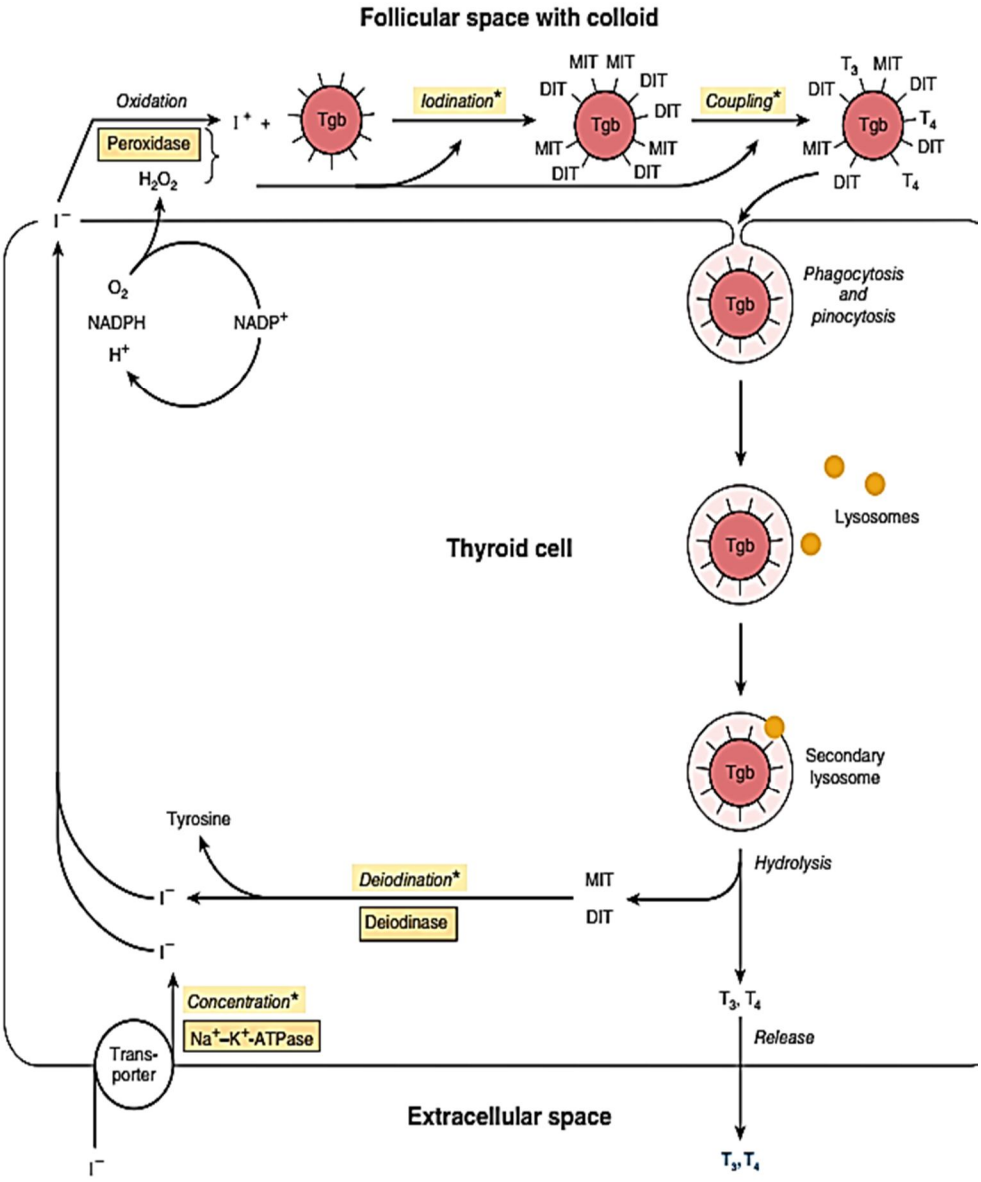
Iodination of the tyrosine residues in thyroglobulin occurs first in the third position of the aromatic nucleus forming “monoiodotyrosine”. It is then iodinated at the fifth position forming “diiodotyrosine.” This is also mediated by the enzyme TPO.

4.COUPLING OF IODOTYROSINE

When the two molecules of diiodotyrosine couples, one molecule of tetraiodotyrosine (T4 : Thyroxine) is formed. MIT combines with DIT to form

T3. Reverse T3 is formed when DIT combines with MIT. In peripheral tissues, the deiodination of the outer ring of T4 by 5' deiodinase produces T3. The human thyroid secretes around 80µg of T4, 4 µg of T3, and 2 µg of reverse T3 per day

MODEL OF IODIDE METABOLISM IN CELL



Pic courtesy : "Harper's illustrated biochemistry 28th edition"

TRANSPORT

About 99% of the iodothyronine (T4 and T3) is protein bound. Remaining 1% is unbound or free form. Only the protein bound form is active.

Proteins binding the thyroid hormones are:

1. Thyroxine binding globulin. (TBG)
2. Thyroxine binding prealbumin also known as “transthyretin”
3. Albumin

SIGNIFICANCE OF THYROID PEROXIDASE[13]

TPO enzyme catalyzes almost the entire metabolism of thyroid hormone synthesis. It is a hemoprotein. It requires many co factors for its functioning such as iron, selenium and zinc. The role of iron as the cofactor plays the basis for pathophysiology in our study.

METABOLISM OF THYROID HORMONES

The primary step in peripheral metabolism of thyroxine is deiodination. T4 is deiodinated in liver, kidney and many other tissues through which we get T3. Only about 13% of T3 is secreted by the thyroid gland while the remaining 87% is formed during this peripheral conversion.

Three different deiodinases are seen :D1, D2, D3 deiodinase

D1 : Liver, kidneys, thyroid and pituitary

D2: Brain, pituitary and brown fat.

D3: brain and reproductive tissues.

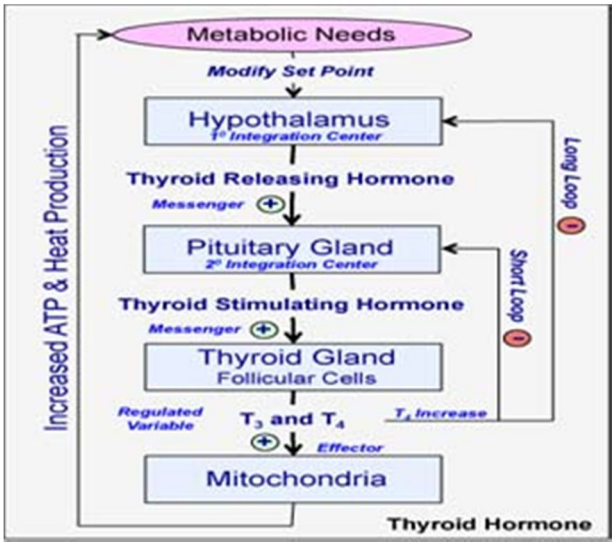
MECHANISM OF ACTION OF THYROID HORMONES[14] :

Thyroid hormones bind to their high affinity receptors intracellularly within the nucleus. Thyroid receptors are : TRα ,TRβ1 and TRβ2[15]. When thyroid hormone binds with the intranuclear receptor, the receptor-ligand complex accumulate within the nucleus. This complex then binds to the specific DNA regulatory sequences : Hormone responsive element [11]. This activates the promoter sequences and increasing the transcription followed by synthesis of more specific proteins i.e., the enzymes. Thus the biological effect occurs.T3 has ten times more affinity than T4 and is biologically more active.

REGULATION OF THYROID HORMONES[9]

Thyroid hormone secretion is regulated at three levels.

1. Hypothalamus: Thyrotropin Releasing Hormone (TRH)
2. Pituitary : Thyroid Stimulating Hormone (TSH)
3. 3.Negative feedback by the thyroid hormones.



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THYROTROPIN RELEASING HORMONE

Thyrotropin releasing hormone is secreted by the hypothalamus. It is a tripeptide (pyro-glutamyl-histidyl-proline amide). TRH stimulates the synthesis of TSH from anterior pituitary. TRH exerts its action by binding to its receptors in the anterior pituitary. This in turn activates the second messenger system , phospholipase C which causes release of inositol triphosphate and diacyl glycerol which releases calcium thus activating protein kinases thereby promoting the release of TSH from anterior pituitary[16].

THYROID STIMULATING HORMONE :

TSH produced by anterior pituitary is also known as thyrotropin . It is a glycoprotein with a molecular weight of about 28kDa. It shares the α subunit with LH ,FSH, hCG. The β subunit is unique to these hormones. Its main function is to promote the synthesis of T4 and T3 . It is the physiological marker for thyroid hormone action.

The mechanism by which it promotes thyroid hormone synthesis are :

1. Increased proteolysis of thyroglobulin
2. Increased activity of the iodide pump
3. Increasing tyrosine iodination
4. Increasing the size as well as secretory activity of thyroid follicles.
5. Changing the thyroid cells from cuboidal to columnar thereby increasing overall secretory activity of thyroid gland mediated by cAMP [16]

   Normal serum TSH levels : 0.3 to 4.2 μIu/mL

THYROXINE and TRIIODOTHYRONINE[17]

Plasma binding proteins have high affinity for the thyroid hormones. Because of this, they are released very slowly particularly T4. Thyroxine is released every 6 days to the cells whereas T3 is released daily. The hypothalamo – pituitary- thyroid axis is responsible for the blood homeostasis of T4 and T3.

KINETICS OF THYROID HORMONE : T3 and T4

S.No.	KINETIC VRIABLE	T4	T3
1	Volume of distribution	1L	40L
2	Extrathyroidal pool	800 µg	54µg
3	Daily production	80 µg	30 µg
4	Fractional turnover per day	10%	60%
5	Metabolic clearance per day	1.1L	24L
6	Half life	7 days	1 day
7	Amount bound	99.96%	99.6%
8	Biological potency	Less potent	More potent
9	Oral absorption	75 to 90%	95%
10	Serum hormone levels	TOTAL 5 to 12 µg/dL FREE 0.9 to 1.7ng/dL	TOTAL 80 to 220 µg/dL FREE 3.2 to 6.8pmol/L

FEEDBACK EFFECT OF THYROID HORMONES ON TRH & TSH

The day to day homeostasis of thyroid hormones is by the negative feedback on TRH & TSH. When thyroid hormone synthesis rate > 1.75 times of the normal, TSH secretion almost becomes nil. Thyroid hormones exerts its effect directly on anterior pituitary as well as on hypothalamus.

Thus excess thyroid hormone inhibits TSH and TRH and vice versa happens in case of thyroid insufficiency.

EFFECTS OF THYROID HORMONES

CELLULAR METABOLISM	Increases mitochondria Increases glucose absorption, gluconeogenesis, glycogenolysis Increases lipolysis & protein synthesis Increases the BMR Increases O_2 consumption
CARDIOVASCULAR SYSTEM	Increases cardiac output Increases heart rate Increases heart strength Increases tissue blood flow Increases respiration
CENTRAL NERVOUS SYSTEM	Increases rapidity of cerebration promoting normal brain development
GROWTH	Responsible for skeletal and bone development
SEXUAL DEVELOPMENT	Lack of thyroxine in women : menorrhagia, polymenorrhea In men : lack of libido
EFFECT ON OTHER ENDOCRINE GLANDS	Stimulates pancreas to secrete insulin Stimulates PTH

HYPOTHYROIDISM : CLASSIFICATION[18]

PRIMARY HYPOTHYROIDISM

It occurs when a disease or treatment destroys the thyroid gland resulting in defective hormone synthesis. It can be of autoimmune origin which may be associated with goiter (hashimoto's or goitrous thyroiditis) or at the end stage, with minimal residual tissue (atrophic thyroiditis)[16] or can be of iatrogenic which develops usually following radioiodine therapy.

SECONDARY HYPOTHYROIDISM

It is due to the disease of pituitary or hypothalamic disorders because of inadequate or absent secretion of TRH or TSH from hypothalamus or anterior pituitary correspondingly resulting in defective thyroid hormone synthesis indirectly.

SUBCLINICAL HYPOTHYROIDISM

Patients who present with bnormal T3 and T4 levels along with raised TSH levels are said to have subclinical hypothyroidism. The thyroid hormones are maintained to normal levels for sometime due to the stimulation of TSH from anterior pituitary. These patients will have very mild to no symptoms.

CLINICAL / OVERT HYPOTHYROIDISM

Patients who present with below normal T3 and T4 levels along with raised TSH levels are said to have clinical / overt hypothyroidism cases. These patients present with sever clinical signs and symptoms of hypothyroidism.

CONGENITAL HYPOTHYROIDISM [19]

Causes include: thyroid gland dysgenesis in 80 to 85 % , inborn defects in thyroid hormone synthesis in 10 to 15% , and TSH –R antibody in 5%

Study by A Hawke et al states that congenital hypothyroidism can occur due to the transplacental passage of anti thyroid peroxidase(TPO) antibodies in maternal hypothyroidism[20].

CONDITION	TSH	fT4	fT3
NORMAL LEVELS	0.3 TO 4.2 µIu/mL	0.9 TO 1.7 ng/dL	3.2 TO 6.8 pmol/L
PRIMARY HYPOTHYROIDISM	Increased	Decreased	Decreased
SECONDARY HYPOTHYROIDISM	Decreased	Decreased	Decreased

CLINICAL FEATURES OF HYPOTHYROIDISM

- Lethargy
- Weight gain
- Cold intolerance
- Pallor
- Puffy face and hands
- Bradycardia
- Constipation
- Ascites
- Ileus
- Menorrhagia

IRON DEFICIENCY ANEMIA [21]

Iron deficiency anemia is the most common cause of nutritional anemia worldwide. The main function of body iron is to carry oxygen along with hemoglobin. Many cellular enzymes are dependent on iron for their action. In case of iron deficiency , electron transport and energy metabolism is affected. In RBC's , hemoglobin synthesis is deficient resulting in poor oxygen delivery to the tissues leading to anemia. Around 20 mL of senescent RBC's are cleared off daily and 20mg of iron is recycled for the production of new RBC's. Since iron forms a part of hemoglobin it is highly essential for heme synthesis.

IRON CYCLE IN HUMANS

Two third of body's iron is seen as part of hemoglobin, 15 to 30 % is stored as ferritin and hemosiderin. Absorption of iron takes place in the duodenum. Remaining iron is in myoglobin[21]. The iron absorbed from the diet or released from the ferritin circulates in the form of transferrin. The circulatory form of iron is "transferrin "

Many proteins are involved in the iron metabolism. Some of them are divalent metal transporter-1 (DMT1), ferroportin (FPN1), and transferrin receptors (Tfrs) in association with ferroxidases such as duodenal cytochrome B, ceruloplamin (Cp) and heme carrier protein (HCP1) (22). Absorption pathways differ for the heme – iron and the non heme- iron . This is important because increased absorption of heme iron may result in higher risk to develop type 2

diabetes mellitus[23]. Whereas increased intake of dietary iron is not a risk factor for diabetes mellitus[23].

Absorption occurs in duodenum. Regulation of iron occurs at the absorption level so “it is a one way element”. When iron stores in the body are depleted, absorption is enhanced. When adequate quantity of iron is stored, absorption is decreased. This is called mucosal block theory”[24][25]. Only ferrous form of iron is absorbed. Ferric form is reduced to ferrous form by the enzyme ferric reductase. A protein called DMT -1 carries the ferrous iron from intestinal lumen into the mucosal cell. Within the cell, ferrous is converted to ferric form which binds with apoferritin to form ferritin. Another transport protein called ferroportin then releases the iron from ferritin into the blood stream. In blood it is reoxidised to ferric state and transported by transferrin.

TRANSFERRIN

Transferrin is a bilobed glycoprotein with two iron binding sites. It exists in two forms : diferric (two iron atoms) and monoferric (single). It serves to solubilize the ferric iron in blood. Iron bound transferrin is called as holo-Tf. This holo-Tf is the main source of iron in many cells. Circulation holo-Tf binds to the transferrin receptor and is endocytosed[26].

The turn over time for transferrin bound iron is 60 to 90 mins. It plays a main role in iron distribution because it transfers the iron from intestine to the reticuloendothelial cells, to the erythroid cells for synthesizing hemoglobin. Normally iron binding transferrin turns over at a rate of 6 to 8 times per day. The amount of iron passing through the transferrin pool is 20 to 24 mg/day[27].

TRANSFERRIN RECEPTOR[28,29]

Transferrin delivers iron to the erythroid or non erythroid cells after interacting with a special type of specific receptor known as the soluble transferrin receptor on the surface of the cell membrane. The receptor is a transmembrane protein which shows its expression in cells requiring iron. The receptor comprises two disulfide linked between cysteines monomers 89 and 98, each containing 760 aminoacids and organized into three major portions [30] :

- A large C terminal extracellular domain of 671 aminoacids
- Tarnsmembrane domain with 2 amino acids
- N terminal cytoplasmic domain of 61 amino acids Structure of human transferrin receptor

Image showing transferrin receptor and cleavage of it to yield soluble transferrin receptor protein

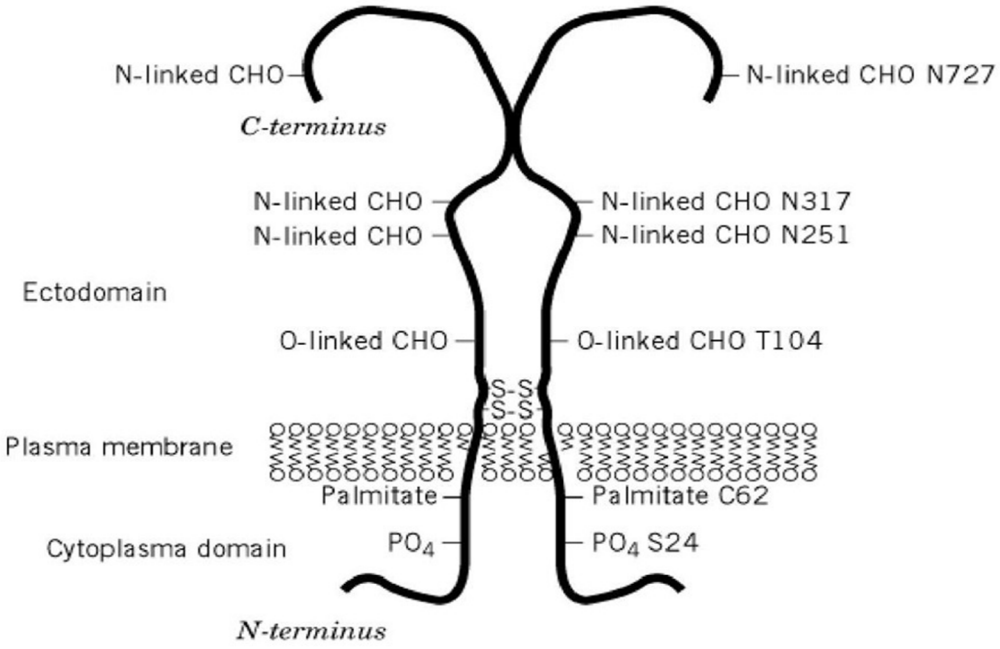


Image courtesy : "https://www.rndsystems.com/resources/articles/solube-transferrin-receptor-stfr"

Image showing cleavage of transferrin receptor to yield soluble transferrin receptor protein

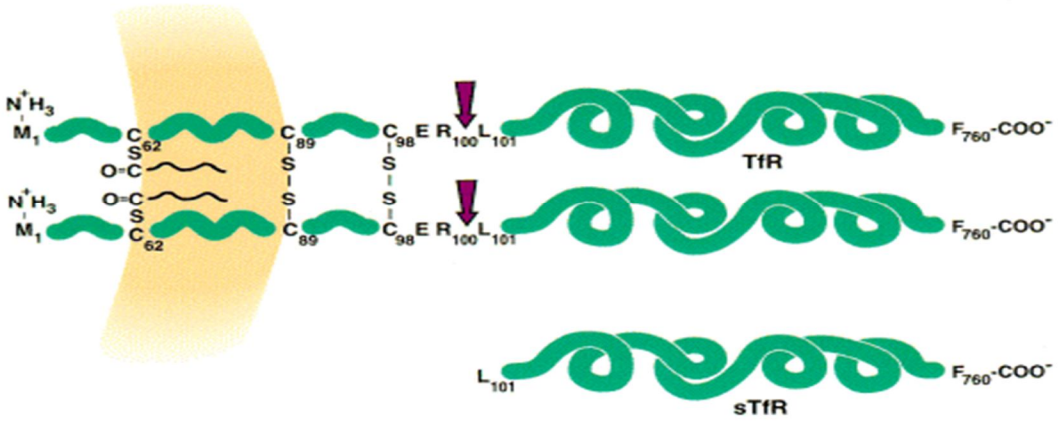
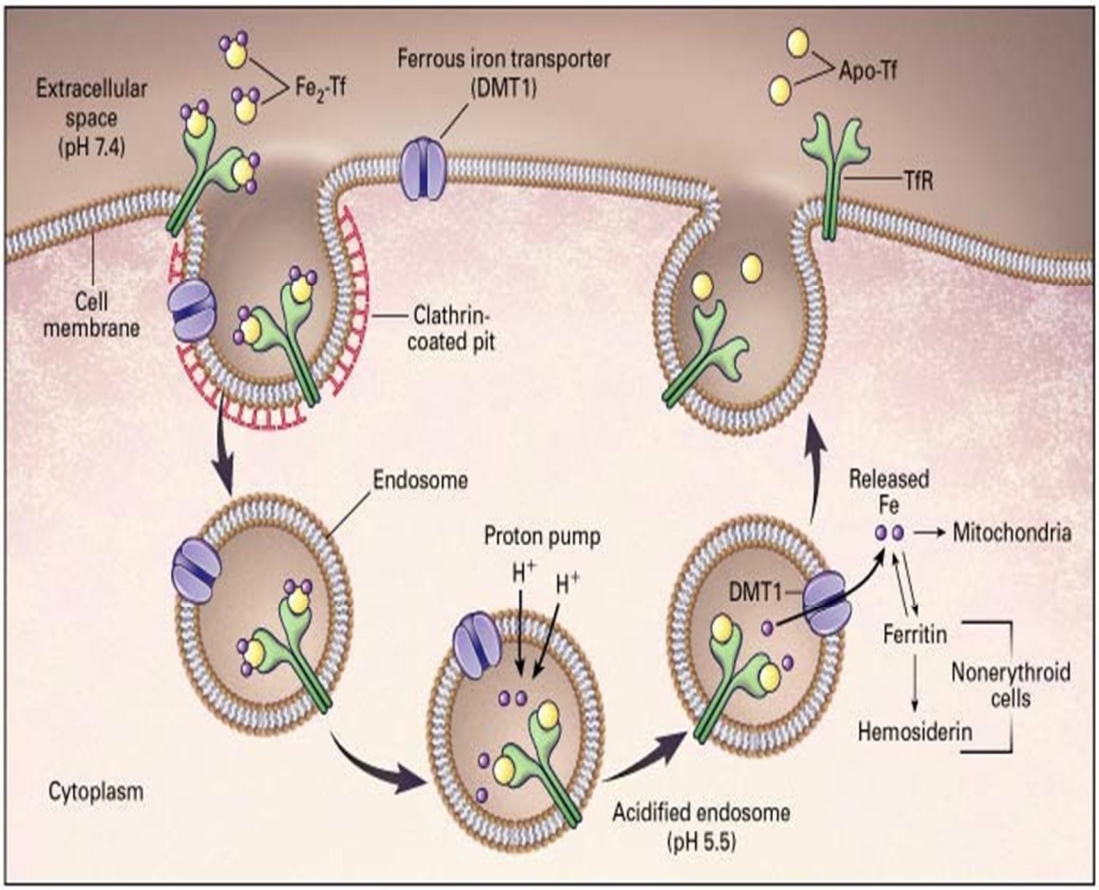


Image courtesy : http://what-when-how.com/molecular-biology/transferrin-receptor-molecular-biology/

The receptor undergoes proteolytic cleavage by a matrix metallo proteinase between arginine -100 and leucine -101 of the extracellular domain gives off soluble transferrin receptor protein (sTfR)[31]. Production of Tf receptor is regulated at the level of mRNA based on the intracellular iron content.[32]. When the level of intracellular iron is low, the iron responsive element increases further facilitating the translation of RNA coding for the ferritin molecule and the reverse happens in case of increased cellular iron concentration. The receptor expression is increased in proximal small intestine especially crypts of the proliferating cells. Erythroblasts are the cells having highest number of receptors (3,00,000 to 4,00,000 /cells). The total body iron stores and the receptor expression is inversely related[33].

When body iron stores are low the receptors expression tends to increase and similarly , there is a down regulation of receptor expression when iron reserves are in plenty[33]. The receptor binds with the serum diferric transferrin and undergoes endocytosis. The complex of transferrin-receptor is internalized by clathrin coated pits. It is then transported to the endosomes. In the endosomes the environment is highly acidic. Due to the low pH , the iron is then released. The iron is then made available for utility while the transferrin- receptor complex is recycled back to the surface of the cell[34].



[The externalization of the iron receptor complex was first described by Pan and Johnstone (35)]. At this point of time , a special type of protein known as the “soluble transferrin receptor protein” sTfR is released into the circulation which can be measured in blood.

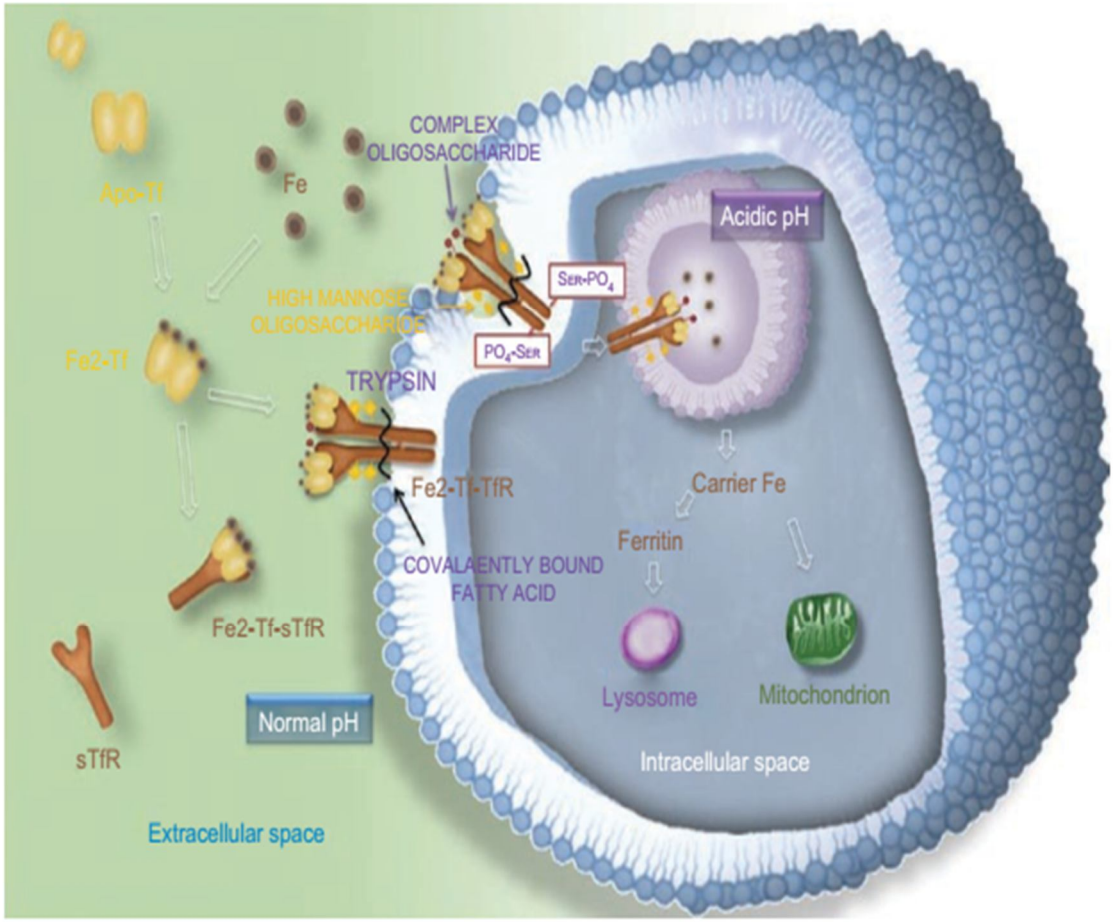


Image courtesy : "Marijn M. Speeckaert et al."

SOLUBLE TRANSFERRIN RECEPTOR PROTEIN (sTfR)

In 1986, Kohgo et al first described the transferrin receptor(29). Soluble transferrin receptor protein is a single polypeptide chain of 85kDa that can be derived from the soluble transferrin receptor. Proteolysis leads to the soluble form of the transferrin receptor(36). It is a truncated form of the receptor without its transmembrane and cytoplasmic domain and circulates bound to transferrin(37). The single polypeptide chain is folded into two lobes C and N which are linked by peptide bonds. Each lobe is divided into two sub domains CI, CII, NI , NII. The iron binding ligands are similar to both the lobes but the rate of iron release differs

in two lobes due to the amino acids surrounding them known as second shell(38). The distribution of the sTfR pool is 27% diferric, 23% monoferric N lobe, 11% monoferrric C lobe and 40% apo. At pH 7.4 diferric Tf binds to the TfR with nanomolar affinity. The binding of monoferric species is 40 times weaker and apo Tf did not compete for binding(38). It is encoded by human TfR gene on chromosome 3. It does not contain the cysteine residues at 89 and 98 position . The concentration of sTfR quantitated has been found to be directly proportional to the number of the transferrin receptors . Plasma sTfR reflects the receptor density on the cells and the number of cells expressing the receptors [31] .

REFERENCE RANGES (39)

The reference range of sTfR for human serum and plasma in Indian population is between 0.3 to 2.9 mg/L [40]. Unlike ferritin it does not vary between sex groups[40].

The cut off value to diagnose iron deficiency anemia is > 2.2 mg/L

CLINICAL CONDITIONS CAUSING ALTERED LEVELS OF sTfR LEVELS[29]

sTfR levels	
INCREASED	Due to decrease in tissue iron Iron deficiency anemia Pregnancy Increased erythropoiesis Autoimmune hemolytic anemia Hereditary spherocytosis β thalassemia/HbE HbH disease Sickle cell disease Polycythemia vera
NORMAL TO INCREASED	Idiopathic myelofibrosis Myelodysplastic syndrome Chronic lymphocytic leukemia
NORMAL	Hemochromatosis Acute and chronic myeloid leukemia Most lymphoid malignancies Solid tumours Anemia of chronic disease
DECREASED	Chronic renal failure Aplastic anemia Post bone marrow transplantation

FERRITIN [41] ,[42]

Free iron is highly toxic to the cells . So the body has developed protective mechanisms to bind iron in various tissues. The storage form of iron is ferritin or hemosiderin. It is composed of both heavy and light chains both of them being encoded by different genes[43]. H chain has 182 amino acids and L chain has 174 amino acids. This proportion may vary from tissues to tissues. Ferritin has a highly conserved three dimensional structure.

Ferritin is present both extra and intracellularly. Apoferritin forms a shell within which iron is stored in the core. Apoferrition is the iron free form and the iron containing is termed as "holoferritin or simply ferritin"[44]. Ferritin has 24 protein subunits arranged in 432 symmetry to give a hollow shell with a 80A° diameter cavity capable of storing upto 4500 Fe(III) as inorganic complex. The shell is an apoferritin shell composing of 24 subunits which are of either Oblong (H) or light (L) chain or the interior is a ferric oxyhydroxide crystalline core [45]. The shell is arranged in a way as to resemble a sphere or snubbled cube consisting of 6 facets[21]. Each facet is formed by a group of 4 apoferritin monomers and a pore in the centre which is 10 A° diameter. The two iron binding sites are present in these pore channel[46].

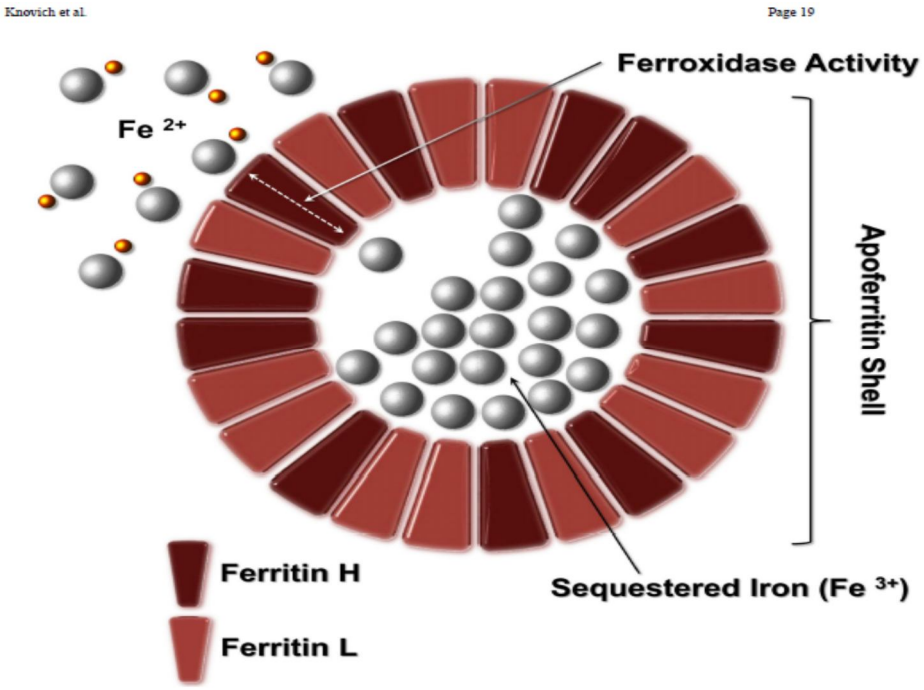


Image courtesy : www.britannica.com

STRUCTURE OF FERRITIN

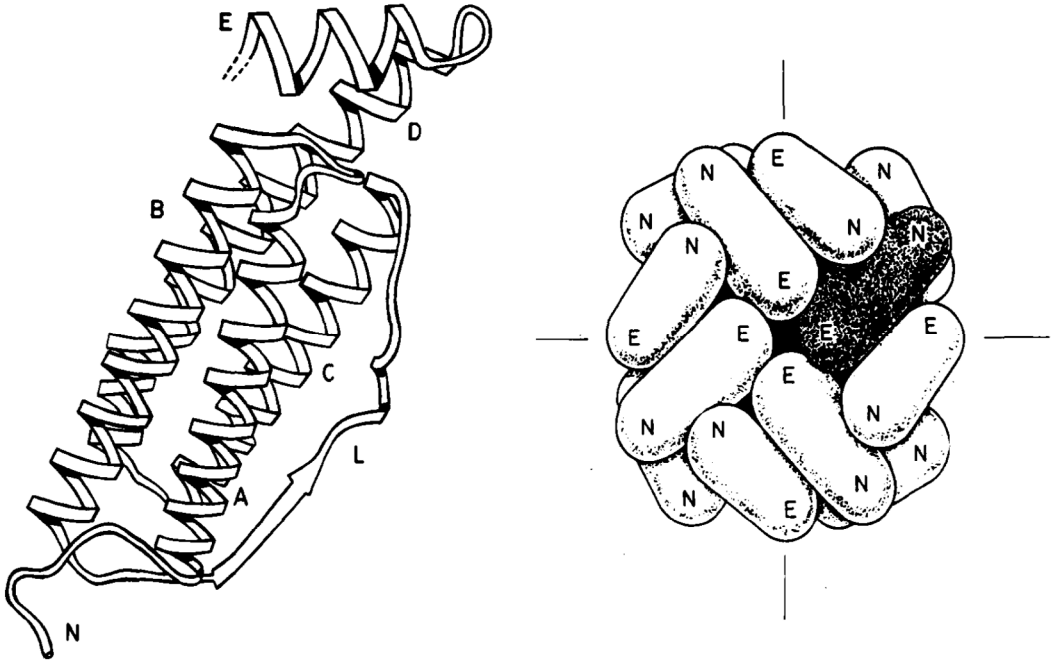


Image courtesy : "The structure and function of ferritin by Pauline m. Harrison"

Figure : 1" Main features are the five helices A to E and long inter helix loop , L. The loop L and the N terminal end, N lie on the outside of the protein shell. Helix E runs from the outside to the inside, so that the C-terminal end of the chain is at the inside surface of the molecule."

Figure : 2 : Twenty-four subunits (sausages) surround the ferritin iron-core (black), which may contain up to 4500 Fe(lll) as "ferrihydrite"

The features of the H and L monomers of apoferritin is given below [44] :

Features	H Monomer	L Monomer
Molecular weight	21,000	18,500
Aminoacid composition	182	175
Chromosome location	11	19
Hydrophilic residues	7	15
Iron binding histidyl unit	Present	Not present
Ferroxidase site	Present	Not present
Nuclear site	Not present	Present
Iron turnover	Takes up more iron readily and retain it less avidly	Very slow
Function	More active in iron metabolism	Long term storage function
Site	Heart	Major iron storage organs mainly liver & spleen

Tissue ferritin vary from H subunit rich (heart and muscle) to L subunit rich (liver and spleen (44),(47).

PLASMA AND TISSUE FERRITIN

BIOCHEMICAL CHARACTERISTICS[48]

Plasma ferritin has a lower affinity than tissue ferritin consequently plasma ferritin has low iron content in contrast to tissue iron [48]

Migration pattern of plasma ferritin in isoelectric focusing is similar to that of apoferritin [48]

High proportion of the plasma ferritin binds to concanavallin A indicating the presence of carbohydrate [48]

Plasma ferritin is synthesized by the rough endoplasmic reticulum whereas intracellular ferritin is produced by the smooth ER.

It is said that both are encoded by different genes[49]

Only ferrous iron is taken up by ferritin, and it is oxidized by a catalytic site on H chain. The core crystal composition of FeOOH contains different amounts of phosphates. In humans it is ferrihydrite ($5Fe_2O_3.9H_2O$) . Ferritin core gives several x ray diffraction lines indicating crystallinity and some of these can be seen as single crystals in electron microscopy. The crystallinity is inversely related to the phosphate content[49]. The steps in iron storage within ferritin involves Fe(II) oxidation , Fe (III) migration and nucleation.

REGULATION OF FERRITIN SYNTHESIS

Ferritin is an intracellular iron storage protein whose synthesis is regulated post-transcriptionally by a mechanism that involves binding of cytoplasmic iron regulatory protein (IRP) to iron-responsive element (IRE) in the 5′ untranslated region of ferritin mRNA[50].

Iron regulatory protein-1 (IRP-1) is a cytosolic RNA-binding protein that is a regulator of iron homeostasis in mammalian cells. IRP-1 binds to RNA structures, known as iron-responsive elements, located in the untranslated regions of specific mRNAs known as iron-responsive elements (IRE) . These IRE regulates the translation or stability of these mRNAs[51] [52].

IREs are 30 nucleotide long RNA motifs that form special stem-loop structures. IREs occur in either the 3'-UTR (untranslated region) or 5'-UTR of an mRNA[52]. Transcripts containing IREs include those encoding[52]:

- Ferritin subunits L and H (expressed in liver and heart, respectively), which mediate iron storage.
- Transferrin, which binds extracellular iron and circulates it in blood plasma.
- Transferrin receptor (expressed on plasma membrane), which controls iron uptake into a cell by binding to iron-bound transferrin.
- Ferroportin, and iron exporter expressed on the surface of gut enterocytes, macrophages and liver cells.

- Divalent metal transporter 1 (DMT1), a ferrous iron transporter that functions in intestinal iron absorption.
- Mitochondrial aconitase
- Succinate dehydrogenase
- Erythroid aminolevulinic acid synthetase, which catalyses the first step in tetrapyrrole synthesis.
- Alzheimer's amyloid precursor protein.

Inhibiting translation: IRP binding IRE within 5'-UTR of mRNA

In iron-deficient cells, the binding of an IRP to an IRE sequence found in the 5'UTR of an mRNA prevents its translation by blocking the mRNA from binding to the ribosome. In iron-replete cells, the absence of IRP binding allows these transcripts to be freely translated. Transcripts that carry IRE in their 5'-UTR include ferritin H and L subunits and aminolevulinic acid synthetase[(52)].

Promoting translation: IRP binding IREs within 3'-UTR of mRNA

Some transcripts contain one or more IRE sequences in their 3'UTR. In iron-deficient cells, the binding of IRPs to these 3' IRE sequences protects them from endonuclease cleavage by blocking nuclease attack, thereby prolonging their half-life and enabling them to be freely translated.

In iron-replete cells, the absence of IRP binding makes these transcripts susceptible to endonuclease attack and subsequent rapid degradation. Transcripts carrying IREs in their 3'-UTR include the transferrin receptor (5 copies) and the DMT1 transporter[52].

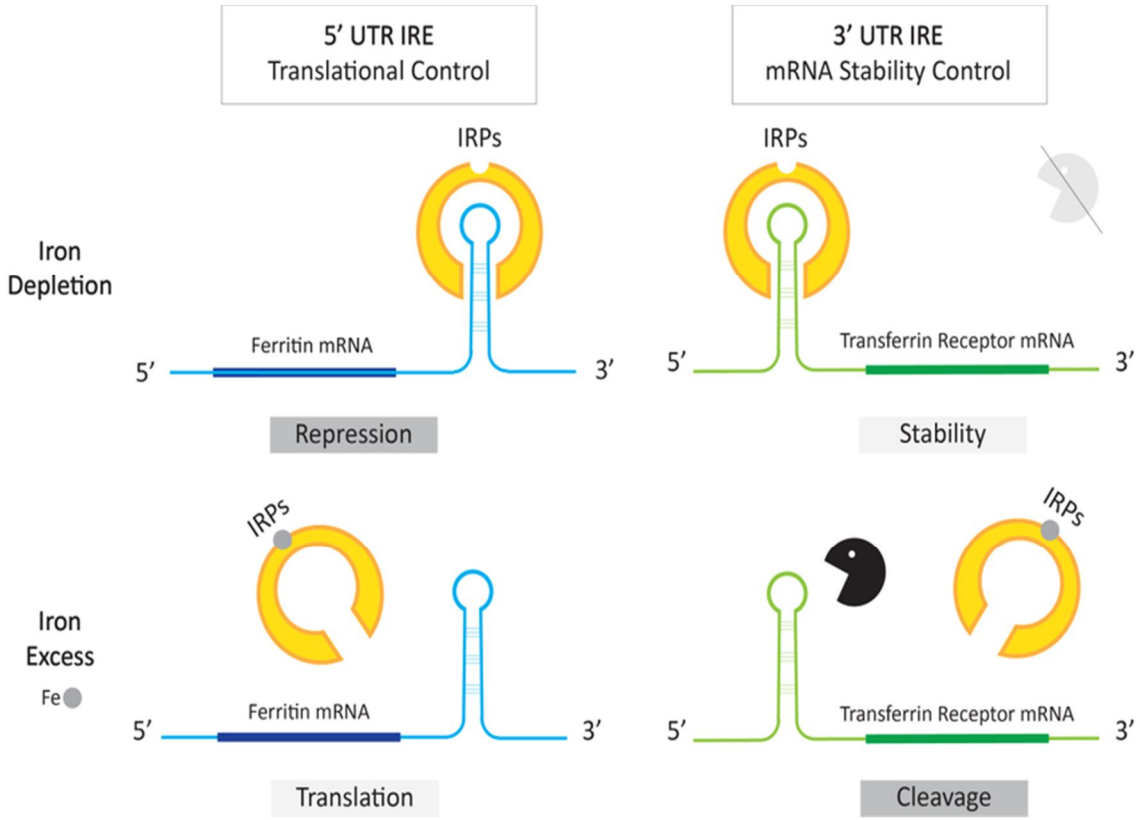


Image courtey : "https://www.researchgate.net/figure/Illustration-of-IRP-IRE-regulatory-system'

Release of ferritin directly leads to potential toxicity which is prevented by degrading it in the membrane bound secondary lysosomes. Iron is stored as ferritin mainly in the liver, spleen and bone marrow. Similarly hemosiderin can also be seen in these areas. In electron microscopy iron can be visualized as tiny dense particles dispersed in the cytoplasm [21].

HEMOSIDERIN

Insoluble, aggregated form of partially deproteinated ferritin is known as hemosiderin. It contains mainly aggregates of the FeOOH core crystals. Due to the smaller surface/volume ratio of hemosiderin , iron will be released only slowly. It has a higher iron content and slower turnover rate than ferritin. Together with ferritin it can be visualized in electron microscopy as membrane bound bodies : siderosomes derived from secondary lysosomes[21].

REFERENCE RANGE FOR SERUM FERRITIN LEVELS :

	ng/mL	µg/L
NEWBORN	25 to 200	25 to 200
1 MONTH	200 to 600	200 to 600
2 TO 5 MONTHS	50 to 200	50 to 200
6 MONTHS – 15 YEAR	7 to 140	7 to 140
ADULT MAN	20 to 250	20 to 250
ADULT WOMAN	20 to 200	20 to 250
IRON OVERLOAD ADULT MAN ADULT WOMAN	 >400 >200	 >400 >200

Source : Textbook of clinical chemistry and mlecular diagnostics by Tietz…

CLINICAL SIGNIFICANCE OF FERRITIN[53],[54]

Usually the level of ferritin in blood is very low. It is the most simple test for estimating the iron stores It is used to

1.To diagnose iron deficiency anemia.

Ferritin is done after evaluating hemoglobin, hematocrit using CBC and a peripheral smear . Ferritin levels are found to be low in IDA and decline even before changes in hemoglobin, red cell size and serum iron is manifested.

2. Depletion of iron stores

Serum ferritin <15µg/L indicates depletion of iron stores. The patient may or may not have anemia. This cut off is valid only for patients without any concomitant disease that may affect the ferritin levels. A normal ferritin does not exclude iron deficiency.

3.In the differential diagnosis of hypochromic microcytic anemia(55)

In hypochromic anemia not caused by iron deficiency (thalassemia, sideroblastic anemia , tumour or infection related) ferritin level is normal or elevated but not low.

4.To monitor the response to iron therapy

In patients with iron deficiency, before starting the therapy serum ferritin is measured to give an idea for the patients body iron reserves. It is definite to measure ferritin to check the improvement in therapy. It should be maintained

within the normal reference range. It is mandatory to do ferritin measurements periodically.

5. Iron overload(56)

Ferritin concentration above 400µg/L is suggestive of iron overload. Further investigations are required to investigate this and the cause should be determined.

1. Hereditary hemochromatosis
2. Secondary iron overload (secondary to repeated blood transfusion)
3. To monitor iron mobilization therapy (deironing)

During monitoring the treatment for HH, ferritin should decrease in response to reduction in iron stores.[55] Being an acute phase reactant, ferritin is affected by chronic disease and inflammation[31] The plasma ferritin levels can be increased in various conditions as given below and can mask the iron deficiency status:

- Inflammation (acute and chronic)
- Significant tissue destruction
- In case of hepatic cell damage such as hepatitis in which the elevated levels of ferritin makes it difficult in diagnosing hereditary hemochromatosis (HH)
- Alcoholic liver diseases (acute phase response or due to the release of ferritin from damaged hepatocytes)

- Therapy with iron supplements
- Heterophile antibodies in human serum can react with reagent immunoglobulins interfering with immunoassays.

Plasma ferritin should always be correlated with clinical and pathological findings. In hereditary hemochromatosis, ferritin is less sensitive than serum iron, TIBC and % transferrin saturation which provides the earliest indicator of HH(57). Measurement of iron in liver biopsy remains the gold standard for diagnosis.

CLINICAL CAUSES FOR CHANGE IN SERUM FERRITIN LEVELS

DECREASED	INCREASED
Iron deficiency anemia Ascorbate deficiency Hypothyroidism	Fever Acute infection Rheumatoid arthritis Chronic inflammatory conditions Hyperferritinemia with cataracts Hereditary hemochromatosis Hodgkin’s lymphoma Leukemia Multiple blood transfusions GRACILE syndrome Acerrloplasminemia Atransferrinemia

STfR- FERRITIN INDEX [28] ,[58], [59], [37]

Many literatures show the evidence that a new index called sTfR /log Ferritin is an evolving tool to diagnose iron deficiency anemia with greater accuracy especially when the levels of ferritin or sTfR are inconclusive. Since there is an inverse relationship between ferritin and sTfR in iron deficiency (increase of sTfR and decrease in ferritin), the ratio between these two parameters , sTfR/log of ferritin ratio helps in improving the diagnostic accuracy of iron deficiency anemia thus strongly differentiating it from anemia of chronic disease(31). This index provides an estimate of the body iron over a wide range of normal and depleted iron stores(60). The normal reference range for the sTfR/log ferritin index in Indian population is : 0.16 to 1.8 (61).However anemia is clinically manifested when this ratio exceeds 3.1[62].

FERRITIN AND sTfR IN IRON DEFICIENCY ANEMIA

Ferritin levels are decreased in iron deficiency anemia [48]. It detects the fall in iron stores at an earlier stage[48]. but since it is an acute phase reactant , low ferritin levels alone may not be equivocal to diagnose iron deficiency[44].

Measurements of circulating sTfR has been used as a routine indicator for the rate of erythropoiesis and an indicator for iron deficiency[29]. The uptake of iron by the cells is under the control of expression by transferrin receptor. If the ferritin levels goes below 15µg/L it indicates the depletion of iron stores .So more amount of transferrin receptor is expressed.

The affinity of the transferrin to the receptor is dependent on the loading state of the receptor. As explained previously, majority of these receptors are located on the erythropoietic cells. The sTfR reflects the TfR level which is a measure of the body iron stores. Also in contrast to ferritin , sTfR concentration is not affected by acute phase reactions, acute liver function disorders, or malignant tumours. When iron deficiency exists, the sTfR concentration rises even before the significant decrease of hemoglobin concentration[(63)].

Therefore "sTfR reflects the body's functional iron status whereas ferritin reflects the iron storage status"[(63)].

"In case of iron deficiency anemia , ferritin levels decrease and the sTfR levels increases."

CONDITION	**sTfR LEVELS**	**FERRITIN**	**sTfR/log Ferritin**
Iron deficiency anemia	INCREASES	DECREASES	INCREASES

?A MUTUAL RELATIONSHIP BETWEEN IRON DEFICIENCY ANEMIA AND HYPOTHYROIDISM : PATHOGENESIS

It has been explained elaborately that the key enzyme involved in thyroid metabolism is thyroperoxidase. This enzyme is dependent on many cofactors for its synthesis . It is a heme containing enzyme. It is highly dependent on iron [64]. Thus it is obvious that iron deficiency can result in the poor performance of thyroperoxidase enzyme. This affects the synthesis of the thyroid hormones resulting in hypothyroidism.. [64,65], [7]

It is also shown that iron deficiency significantly affects efficiency of 5' deiodinase enzyme [65].This enzyme is involved in the peripheral conversion of T4 to T3 and also decreases the TSH response to thyroid releasing hormone (TRH)[2]. This is the most accepted cause for iron deficiency resulting in hypothyroidism.

Lack of stimulation of the erythroid colony development by thyroid hormones in hypothyroidism decreases the erythropoietin levels. So there is a reduction of oxygen distribution to the tissues which further affects the iron metabolism. This is one of the cause for hypothyroidism resulting in iron deficiency anemia.

Patients with iron deficiency anemia presents with symptoms such as palpitation, increased heart rate, menstrual disturbances, anxiety[66]. The fact that iron deficiency anemia causing hypothyroidism is to be kept in mind while

supplementing thyroxine to hypothyroid patients with iron deficiency anemia. Because thyroxine stimulates sympathetic activity so the symptoms of sympathetic over activity may still worsen for these patients.

Also thyroxine supplementation helps in improving erythropoiesis thus erythropoietin levels increase. Increased erythropoiesis results in increased production of RBC which require still more iron in already iron deficient patients. This further increases the iron deficiency anemia. This is the second cause explaining hypothyroidism and iron deficiency anemia [67].

Thus hypothyroidism may be a cause and effect for iron deficiency anemia or the other way round. This explains the mutual relationship between the thyroid function and the iron status.

Aim & Objectives

AIM & OBJECTIVES

AIM OF THE STUDY

1. To study the iron status among hypothyroid patients
2. To correlate the ferritin & soluble transferrin receptor levels along with thyroid profile in hypothyroid patients

OBJECTIVES

1. To estimate thyroid hormone levels in all the selected subjects
2. To diagnose people with hypothyroidism
3. To classify the subjects into euthyroid and hypothyroid groups
4. To assess the iron status in healthy controls and hypothyroid patients using:

    Measurement of CBC

    Performing peripheral smear

    Estimation of serum ferritin levels

    Estimation of sTfR levels

    Calculation of sTfR – Ferritin index

    Which is given by the formula : sTfR/ log of ferritin
5. To classify subjects into normal and iron deficiency anemia patients
6. To correlate iron status with the thyroid hormones among hypothyroid patients

Materials & Methods

MATERIALS AND METHODS

The study protocol was approved by the Institutional Ethics committee of Madras Medical College, Chennai and a copy of it has been enclosed.

STUDY DESIGN

Cross sectional study

STUDY PERIOD

May 2017 to December 2018

SUBJECT SELECTION

100 subjects were selected among which

50 subjects are hypothyroid and

50 subjects were healthy euthyroid controls.

INCLUSION CRITERIA

GROUP A : CASES

1. 15 to 60 years of age
2. Newly diagnosed hypothyroid patients
3. Not treated with any thyroid medication or lipid lowering drugs
4. Ambulatory out patients attending endocrinology outpatient department.
5. Hypothyroidism is diagnosed based on the TSH, FT3 and FT4 values

GROUP B : CONTROLS

1. Healthy volunteers of 15 to 60 years of age

EXCLUSION CRITERIA

1. Pregnant or lactating females
2. Patients receiving iron supplements
3. Patients receiving drugs which may affect the thyroid status
4. Patients with acute illness
5. Patients diagnosed with diabetes mellitus
6. Patients with cardiovascular disease
7. Patients with thyroid malignancies
8. Patients with associated chronic medical conditions

SAMPLE COLLECTION

5 mL of blood sample was collected from all subjects. 3 mL of the sample was transferred to serum tube and 2 mL to K_2 EDTA tubes. After adequate clotting, serum tube is centrifuged. The serum which gets separated in the tube is aliquoted . it is used for further testing of thyroid profile , ferritin, soluble transferrin receptor levels. From the EDTA tubes plasma was separated and CBC was analysed. For peripheral blood smear analysis drop of blood was collected by needle prick. The aliquoted sample was stored at - 20◦C

1.ESTIMATION OF SERUM sTfR LEVELS

METHOD - Enzyme Linked Immuno Sorbent Assay (ELISA) : Sandwich using ERBA ELISA washer and Bio rad ELISA reader.

PRINCIPLE

The ELISA wells are pre coated with sTfR monoclonal antibody. Sample containing sTfR is added to the microtitre wells which is precoated with the antibody and then left for incubation. After the period of incubation , biotin conjugated anti human sTfR antibody is added and it binds to the human sTfR. Leave for incubation at 37◦C. After incubation the unbound biotin conjugated anti human sTfR is washed away during a washing step. Streptavidin –HRP is added and binds to the biotin conjugated anti human sTfR antibody. After inubation unbound Streptavidin- HRP is washed away during another washing step. Substrate solution is then added and colour develops.

The intensity of colour developed is in proportion to the amount of human sTfR. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450nm.

REAGENTS

1. Standard solution (4.8 mg/L)
2. Standard Diluent
3. Precoated ELISA plate
4. Streptavidin HRP
5. Stop solution

6. Substrate solution A
7. Substrate solution B
8. Wash buffer concentrate (30x)
9. Biotin conjugated anti – human sTfR antibody

REAGENT PREPARATION

All reagents should be brought to room temperature before use.

WASH BUFFER

Dilute 20ml of wash buffer concentration into deionized water to yield 500ml of wash buffer. If crystals have formed in the concentrate mix gently until the crystals have completely dissolved.

STANDARD PREPARATION

2.4mg/L Standard No.5 120µl Original Standard + 120µl Standard diluents

1.2mg/L Standard No.4 120µl Standard No.5 + 120µl Standard diluents

0.6mg/L Standard No.3 120µl Standard No.4 + 120µl Standard diluent

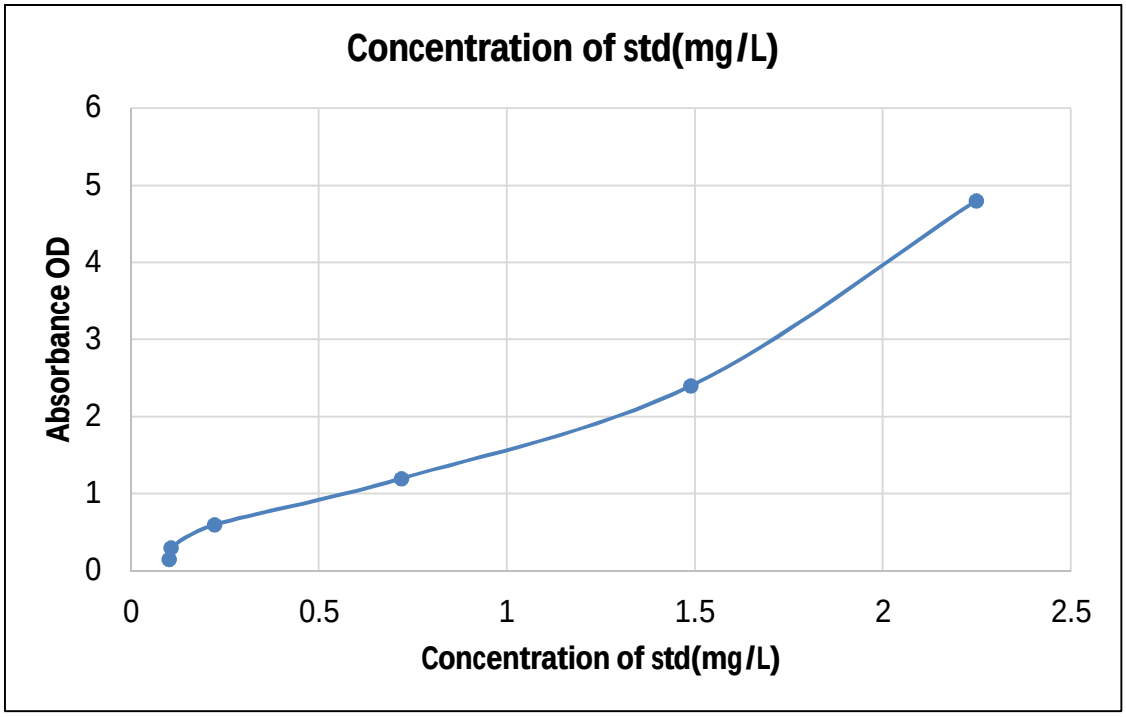
0.3mg/L Standard No.2 120µl Standard No.3 + 120µl Standard diluent

0.15mg/L Standard No.1 120µl Standard No.2 + 120µl Standard diluents

STANDARD	**S5**	**S4**	**S3**	**S2**	**S1**
4.8mg/L	2.4mg/L	1.2mg/L	0.6mg/L	0.3mg/L	0.15mg/L

S.No	Concentration of std (mg/L)	Absorbance (OD)
1.	0.15	0.090
2.	0.3	0.105
3.	0.6	0.221
4.	1.2	0.718
5.	2.4	1.488
6.	4.8	2.247

STANDARD GRAPH OF sTfR ASSAY



ASSAY PROCEDURE

1) Blank well: No sample, anti sTfR antibody labeled with biotin or streptavidin-HRP is added to comparison blank well except chromogen solution A & B and stop solution while taking the same steps that follow.
2) Standard solution well:

   Add 50μl standard and streptomycin-HRP 50μl (biotin antibodies have united in advance in the standard so no biotin antibodies are added.)
3) Sample well to be tested: Add 40μl sample and then 10μl sTfR antibodies, 50μl streptavidin-HRP. Then cover it with seal plate membrane.
4) Washing: Remove the seal plate membrane carefully, drain the liquid and shake off the remaining liquid. Fill each well with washing solution. Drain the liquid after 30 seconds' standing.

   Then repeat this procedure five times and blot the plate.

   Shake gently to mix them up. Incubate at 37℃ for 60 minutes.
5) Color development: Add 50μl chromogen solution A firstly to each well and then add 50μl chromogen solution B to each well as well. Shake gently to mix them up. Incubate for 10 minutes at 37℃ away from light for color development.
6) Stop: Add 50μl Stop Solution to each well to stop the reaction (the blue color changes into yellow immediately at that moment).
7) Assay: Take blank well as zero, measure the absorbance (OD) of each well one by one under 450nm wavelength, which should be carried out within the 10 minutes after having added the stop solution.

SENSITIVITY OF THE ASSAY

Minimum sTfR detectable limit is： 0.005mg/L.

Assay range：0.01mg/L→4mg/L.

2. ESTIMATION OF SERUM FERRITIN LEVELS

METHOD

Enzyme Linked Immuno Sorbent Assay (ELISA), non competitive sandwich performed with ERBA ELISA washer and Bio rad reader.

PRINCIPLE

Ferritin in standard and test samples are captured by anti ferritin antibody coated on microtitre wells. Then a second antibody to ferritin conjugated with horse radish peroxidase to is added which binds with the immobilized ferritin, thus forming a sandwich of ferritin antigen between two anti- ferritin antibodies. Then substrate to the enzyme horse radish peroxidase is added resulting in the formation of a coloured complex.

The intensity of colour produced is directly proportional to concentration of ferritin in the sample. The absorbance is measured at 450 nm in an automated microplate reader.

REAGENTS

Reagents are procured from DIA.METRAS.r.l.company

1. Standard solution 6 vials
2. Standard Diluent
3. Control 1Ml
4. Precoated ELISA plate
5. Streptavidin HRP
6. Stop solution
7. TMB Substrate solution
8. Wash buffer concentrate (30x)

PROCEDURE :

1. All reagents and samples should be brought to room temperature.
2. Standards are ready to use.
3. Add 20µL of standard to the corresponding standard wells. Then add 20µof sample to all wells
4. Add 100 µL of conjugate to each well.
5. Cover the wells and incubate for 1 hour at room temperature(22 to 28◦C) .
6. Remove the contents from each well after incubation
7. Wash the wells 3 times with 300 µL of diluted wash solution
8. During each washing step, the plae should be shaken gentlyfor 5 seconds and remove excess solution by tapping the inverted plate on an absorbent paper towel
9. Then add 100 µL TMB substrate to each well
10. Incubate for 10 minutes at room temperature in the dark

11. Then add 100 µL of stop solution
12. Shake the plate gently

Absorbance is read at 450nm against a reference wavelength of 620nm to 630nm.

SENSITIVITY OF THE KIT

The lowest detecteble concentration of ferritin that can be distinguished from the calibrator 0 is 0.04µg/L at the 95% confidence

3.ESTIMATION OF TSH

METHOD

Electro chemiluminescence immunoassay(ECLIA) – sandwich assay in COBAS 6000 automated hormone analyser.

PRINCIPLE

First incubation : 50µL of sample , a biotinylated monoclonal TSH-specific antibody and a monoclonal TSH- specific antibody labeled with a ruthenium complex react to form a sandwich complex.

Second incubation : after addition of streptavidin coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.

The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode.

Unbound substances are then washed away. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier

Results are determined via a calibration curve generated by 2 point calibration

REAGENTS:

Reagents are procured from Roche diagnostics limited

1. Streptavidin coated microparticles 0.72mg/Ml
2. Biotinylated anti – TSH- mouse Antibody 2mg/L
3. Monoclonal anti-TSH antibody labeled with ruthenium complex 1.2mg/L
4. Phosphate buffer 100mmol/L

SENSITIVITY OF THE KIT

0.014µIU/Ml with an intermediate precision CV of ,less than or equal to 20%

ESTIMATION OF fT4

METHOD

Electro chemiluminescence immunoassay(ECLIA) – competitive assay in COBAS 6000 automated hormone analyser.

PRINCIPLE

First incubation : 15µL of sample and a T4 specific antibody labeled with a ruthenium complex.

Second incubation : after addition of biotinylated T4 and streptavidin coated microparticles, the still free binding sites of the labeled antibody become occupied, with formation of an antibody-hapten complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin.

The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then washed away. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier

Results are determined via a calibration curve generated by 2 point calibration

REAGENTS:

Reagents are procured from Roche diagnostics limited

1. treptavidin coated microparticles 0.72mg/Ml
2. Biotinylated T4 2.5ng/mL
3. Polyclonal anti T4 antibody labeled with ruthenium complex 75ng/mL
4. Phosphate buffer 100mmol/L

MEASURING RANGE : 0.3 to 100 ng/dL

ESTIMATION OF fT3

METHOD

Electro chemiluminescence immunoassay(ECLIA) – competitive assay in COBAS 6000 automated hormone analyser

PRINCIPLE

First incubation : 15µL of sample and a anti T3 specific antibody labeled with a ruthenium complex

Second incubation : after addition of biotinylated T3 and streptavidin coated microparticles, the still free binding sites of the labeled antibody become occupied, with formation of an antibody-hapten complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin

The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then washed away. Application of a voltage to the

electrode then induces chemiluminescent emission which is measured by a photomultiplier

Results are determined via a calibration curve generated by 2 point calibration

REAGENTS:

Reagents are procured from Roche diagnostics limited

1. Streptavidin coated microparticles 0.72mg/mL
2. Biotinylated T3 2.4ng/mL
3. Monoclonal anti T3 antibody labeled with ruthenium complex 18ng/mL
4. Phosphate buffer 100mmol/L

Measuring range : 0.4 to 50 pmol/L

ESTIMATION OF HEMOGLOBIN

METHOD

Sysmex automated cell counter

PRINCILPE

Through capillary action the blood sample is aspirated by the analyser. Hemoglobin is estimated based on the measurement of absorbance of a Hb/HbO2 isoelectric point. The absorbance is measured at two wavelengths at 506nm and 880nm.

Measurement at bichromatic wavelength is to decrease the interferences like turbidity in the sample, interference by hemoglobin variants like methemoglobin and carboxyhemoglobin.

SENSITIVITY

For 7.3g/dL , CV is 0. 82%

Statistical Analysis

STATISTICAL ANALYSIS

- The data was analysed using SPSS (Statistical Package for Social Science) Version16.01. The data collected was tabulated in excel sheet and analyzed.
- Continuous variables were presented as mean ± SD (standard deviation) and categorical variables were represented as frequencies and percentages.
- Gender was analyzed by Chi square test.
- Age, TSH, FT3, FT4, Serum ferritin, serum soluble transferrin receptor levels and calculated stfr/log ferritin ratio mean was compared between study groups by student t test.
- Pearson Correlation was used to find out the relationship between variables
- All the Statistical results were considered to be significant at P value $\leq$ 0.05.

Results

MASTER CHART - CASES										
S. NO	AGE	SEX	Hb in CBC	PERIPHERAL SMEAR	TSH µlu/mL	T3 pmol/L	T4 ng/dL	FERRITIN	sTfR mg/L	sTfR / log ferritin
1	35	F	10.1	Microcytic hypochromic anemia	4.5	2.6	0.7	15	4.3	3.66
2	21	F	10.9	Mild microcytic hypochromic anemia	6.6	2.8	0.8	13	4.2	3.77
3	39	M	9.8	Moderate microcytic hypochromic anemia	25.3	2.5	0.8	12	4.2	3.89
4	18	F	10.2	Microcytic hypochromic anemia	5	2.7	0.6	11	4	3.84
5	46	M	11.4	Moderate microcytic hypochromic anemia	44.2	2.9	0.2	14.5	4.2	3.62
6	35	F	7.4	Moderate microcytic hypochromic anemia	100	2.1	0.1	11	3.9	3.74
7	59	M	8.2	Moderate microcytic hypochromic anemia	17.6	2.8	0.5	6.2	3.6	4.54
8	53	F	8.6	Moderate microcytic hypochromic anemia	33.8	2.5	0.3	7.3	3.9	4.52
9	35	F	9.3	Mild microcytic hypochromic anemia	100	2.6	0.3	10.3	3.2	3.16
10	34	M	10	Mild microcytic hypochromic anemia	23.5	2.7	0.6	12.3	3.4	3.12
11	32	F	7.6	Moderate microcytic hypochromic anemia	4.7	2.9	0.4	15	3.8	3.23
12	38	F	11.5	Mild microcytic hypochromic anemia	65.2	2.6	0.5	12.3	3.6	3.30
13	42	F	8.9	Moderate microcytic hypochromic anemia	77.1	2.3	0.3	10.3	3.2	3.16
14	43	M	9	Mild microcytic hypochromic anemia	6.5	2.3	0.6	9.9	3.6	3.62
15	54	F	10.2	Mild microcytic hypochromic anemia	10.2	2.1	0.7	14.3	3.9	3.38
16	56	F	9.3	Mild microcytic hypochromic anemia	79.1	2.6	0.5	11.6	3.8	3.57
17	38	M	10	Mild microcytic hypochromic anemia	6.8	2.6	0.4	8.7	3.2	3.41
18	38	F	8.2	Moderate microcytic hypochromic anemia	77.2	2.6	0.6	15.2	3.9	3.30
19	34	F	10.5	Mild microcytic hypochromic anemia	100	2.5	0.7	14	3.7	3.23
20	37	F	10	Mild microcytic hypochromic anemia	35.6	2.8	0.8	13.6	3.9	3.44
21	23	F	11	Mild microcytic hypochromic anemia	4	3.1	0.9	469.6	0.7	0.26
22	37	F	9.3	Mild microcytic hypochromic anemia	6	2.8	0.4	8.3	3.6	3.92
23	43	F	7.3	Moderate microcytic hypochromic anemia	66.5	2.5	0.3	12.3	3.8	3.49
24	28	M	11	Mild microcytic hypochromic anemia	28.3	2.3	0.8	11.6	3.3	3.10
25	29	F	9	Mild microcytic hypochromic anemia	121	2.6	0.3	12.8	3.5	3.16
26	45	F	10.3	Mild microcytic hypochromic anemia	53	2.9	0.5	12.5	3.5	3.19
27	48	M	9.8	Mild microcytic hypochromic anemia	33.5	2.7	0.4	11.3	3.6	3.42
28	49	M	7.7	Moderate microcytic hypochromic anemia	77.3	2.5	0.6	8.9	2.9	3.05

S. NO	AGE	SEX	Hb in CBC	PERIPHERAL SMEAR	TSH µlu/mL	T3 pmol/L	T4 ng/dL	FERRITIN	sTfR mg/L	sTfR / log ferritin
29	46	M	8.6	Moderate microcytic hypochromic anemia	8.7	4.6	0.5	12.6	3.6	3.27
30	28	M	7.3	Moderate microcytic hypochromic anemia	4.6	5.6	0.4	15.9	3.9	3.25
31	36	F	8.9	Moderate microcytic hypochromic anemia	17.6	1.9	0.5	12.9	3.9	3.51
32	35	F	11.1	Mild microcytic hypochromic anemia	15	2.9	0.3	10.8	3.2	3.10
33	23	F	8.8	Moderate microcytic hypochromic anemia	18.2	2.9	0.7	11.3	4	3.80
34	43	F	11.1	Mild microcytic hypochromic anemia	36	2.8	0.3	12.6	3.9	3.54
35	33	F	12.8	Mild microcytic hypochromic anemia	16.9	1.9	0.5	13.7	4	3.52
36	36	F	9.6	Moderate microcytic hypochromic anemia	10.2	2	0.6	11.9	4.3	4.00
37	37	F	7.7	Moderate microcytic hypochromic anemia	4.9	2.1	2.9	12.3	3.8	3.49
38	35	F	7.2	Megaloblastic anemia	13.3	2.8	0.8	15.5	3.3	2.77
39	44	F	11.2	Megaloblastic anemia	14.3	2	0.3	10.2	3.6	3.57
40	40	F	11.2	Mild microcytic hypochromic anemia	32	2.5	1.1	12.2	3.5	3.22
41	41	F	11.3	Mild microcytic hypochromic anemia	121	2.6	0.5	11.1	3.4	3.25
42	18	F	10	Moderate microcytic hypochromic anemia	6.8	2	0.2	12.1	3.4	3.14
43	20	F	12.1	Mild microcytic hypochromic anemia	5.6	2.3	0.6	13.9	3.6	3.15
44	19	F	9.5	Moderate microcytic hypochromic anemia	12.3	2.6	0.6	11.6	3.7	3.48
45	42	F	12.2	Mild microcytic hypochromic anemia	5.8	2.8	0.5	13.1	3.3	2.95
46	38	M	11.1	Mild microcytic hypochromic anemia	4.8	2.3	0.4	11.1	3.3	3.16
47	41	F	12.3	Mild microcytic hypochromic anemia	4.1	2.1	0.5	12.1	3.4	3.14
48	58	F	11.1	Mild microcytic hypochromic anemia	6.8	2	0.5	11.6	3.8	3.57
49	40	F	12.1	Mild microcytic hypochromic anemia	11.2	2.2	0.3	12.3	3.5	3.21
50	37	M	12.8	Mild microcytic hypochromic anemia	2.7	2.3	0.6	12.6	3.6	3.27

MASTER CHART - CONTROLS										
S. NO	AGE	SEX	Hb in CBC	PERIPHERAL SMEAR	TSH µlu/mL	T3 pmol/L	T4 ng/dL	FERRITIN	sTfR mg/L	sTfR / log ferritin
1	29	F	13.1	Normal study	4.2	5.9	1.5	49	2.1	1.24
2	19	F	12.8	Normal study	4	5.5	1.6	417	2	0.76
3	55	F	12.9	Normal study	4.3	4.5	1.4	137	2	0.94
4	35	F	13	Normal study	4.1	3.5	1.1	43.7	0.5	0.30
5	38	M	13 .1	Normal study	4.1	3.7	1.3	451.1	1.1	0.41
6	36	F	14.6	Normal study	4.1	4.6	1.1	241.13	1.5	0.63
7	48	M	13.6	Normal study	4.1	3.8	1.4	97.5	0.9	0.45
8	28	F	13.8	Normal study	3.9	3.2	1.1	74.2	1	0.53
9	32	F	13.9	Normal study	0.8	5	1.5	83.1	1	0.52
10	33	F	13.2	Normal study	3.9	5.2	0.9	33.2	1.2	0.79
11	52	M	13.4	Normal study	4	5.7	0.5	33.2	1.8	1.18
12	43	F	12.9	Normal study	2.4	3	1.6	109.5	1.5	0.74
13	39	F	13.9	Normal study	1.5	5.1	1.1	62.1	1.4	0.78
14	35	M	14	Normal study	2.2	5.2	1.1	78.1	1.2	0.63
15	46	F	13.7	Normal study	2.9	4.5	1.5	138	0.9	0.42
16	21	F	14.3	Normal study	1.7	3.9	0.9	147	0.9	0.42
17	32	M	15.3	Normal study	2.1	5.2	1.2	458.1	0.8	0.30
18	42	F	13.3	Normal study	1.5	2.8	0.9	392.6	1.1	0.42
19	22	F	14.8	Normal study	2.8	5.3	1.2	112.6	1.6	0.78
20	40	F	13.2	Normal study	2.5	4.2	1.1	584.3	0.3	0.11
21	46	M	15	Normal study	3.8	6.5	0.9	696	0.6	0.21
22	31	F	12.9	Normal study	4.1	6	1.2	715	1.4	0.49
23	39	F	13	Normal study	0.9	4.2	1.1	36.4	1	0.64
24	32	F	13.6	Normal study	1.3	3.9	1.2	25.3	1.2	0.86
25	28	F	13.8	Normal study	1.8	5.2	0.9	111.6	1	0.49
26	36	F	14	Normal study	2	4.3	1.5	128	1.1	0.52
27	38	M	16	Normal study	2.6	5.7	1.6	122.6	1.1	0.53
28	42	F	13.5	Normal study	3.9	4.6	1	27.4	1.4	0.97
29	21	F	13.9	Normal study	3.6	4.2	1.7	60.8	1.3	0.73
30	29	F	13.2	Normal study	1.6	3.6	1.5	188.9	1.2	0.53
31	29	F	13.2	Normal study	0.9	3.9	1.5	260.4	1.3	0.54
32	56	F	13.1	Normal study	0.9	2.7	0.5	51.1	1	0.59
33	26	F	13.9	Normal study	3.5	5.5	1.5	118.5	1.5	0.72
34	32	F	14	Normal study	4	3.2	1.2	135.5	1.3	0.61
35	46	F	13.3	Normal study	3.2	5.6	1.1	30.2	3.2	2.16
36	50	F	13.6	Normal study	4.1	6.5	0.9	406	3.2	1.23
37	33	F	14	Normal study	3.3	5.2	1	18.1	3.5	2.78
38	42	F	15	Normal study	2.9	3.9	0.9	204	1.5	0.65
39	46	F	13.9	Normal study	4.1	6.2	1.5	392	1.413	0.54
40	53	F	12.9	Normal study	3.6	4.5	1.3	534.5	1.5411	0.56
41	56	F	14.1	Normal study	2.6	5.8	1.6	18.9	3.9	3.06
42	33	F	13.6	Normal study	2.1	3.6	1.2	138.3	1.17	0.55
43	31	F	14.2	Normal study	3.2	5.6	1.3	254.5	1	0.42
44	22	F	13.7	Normal study	2.8	4.3	1.6	358.7	0.7	0.27
45	27	F	14.1	Normal study	2.9	5.2	0.7	406	1.1	0.42
46	52	F	13	Normal study	2.8	3.5	1.5	125	1	0.48
47	26	F	14	Normal study	4.3	4.5	1.7	145	1.2	0.56
48	35	M	15.2	Normal study	2.5	4.5	0.9	302	1.8	0.73
49	26	M	14.8	Normal study	5.6	5.5	1.1	240	1.3	0.55
50	40	M	12.2	Relative lymphocytosis	3.9	5.1	1.41	102	2.1	1.05

RESULTS

Table 1 : Distribution of study participants according to age.

Age in years	*Cases*		*Controls*		*Percentage (%)*
	N	*%*	*N*	*%*	
< 20	4	7	1	2	4.5
21-30	6	11.7	12	24.4	18.1
31-40	22	45	21	40.8	42.9
41-50	13	25	10	20.4	22.7
51-60	5	9.8	6	12.2	11
Total	50	100	50	100	100
Mean	37.63		36.48		
Standard deviation	9.7		10.4		
p value	0.28				

The overall mean age of the study participants were 37.07±9.90 years. Most of the study participants were in the age group of 31-40 years.

Figure 1 : Bar diagram showing the frequency of study participants according to age

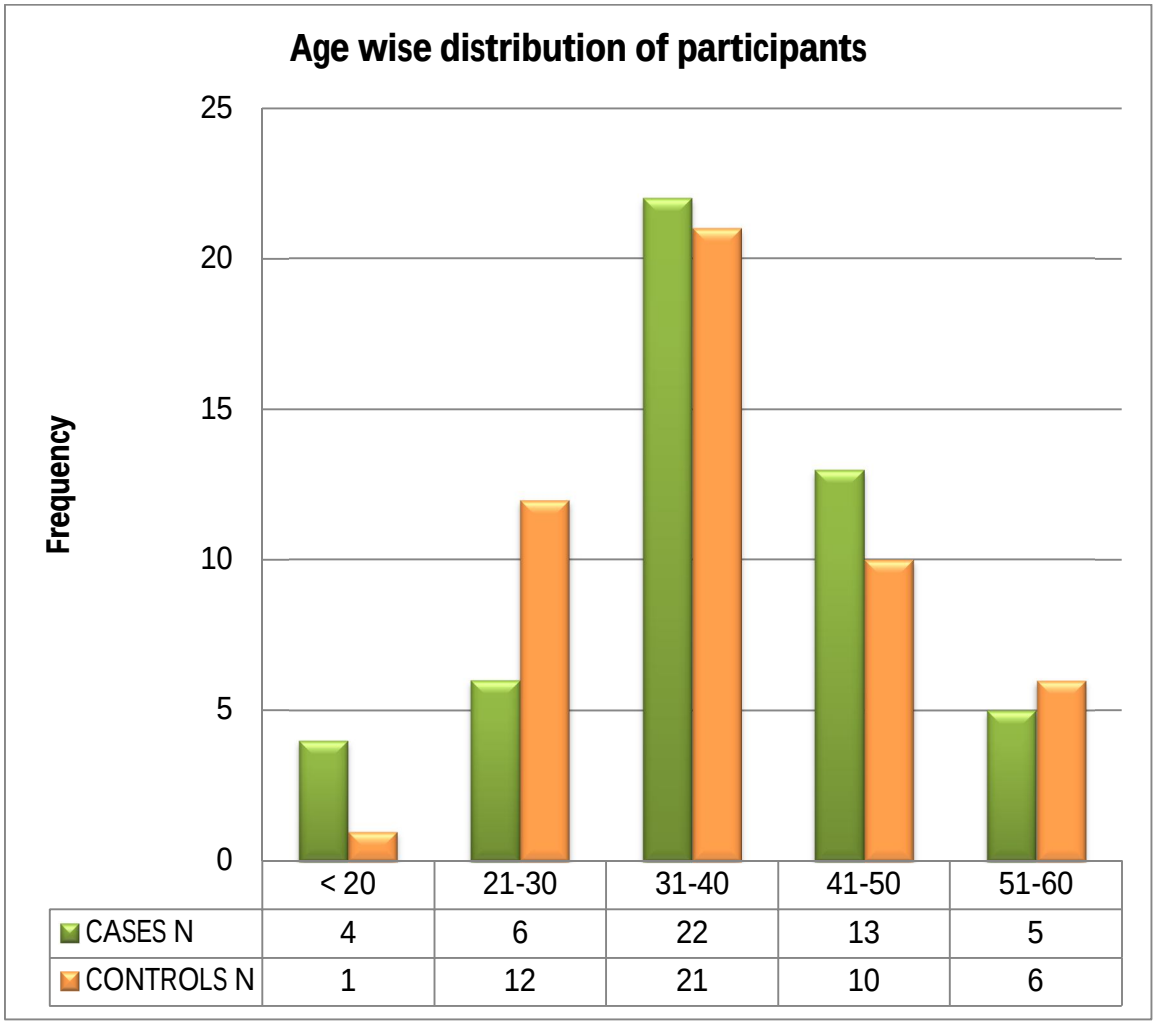
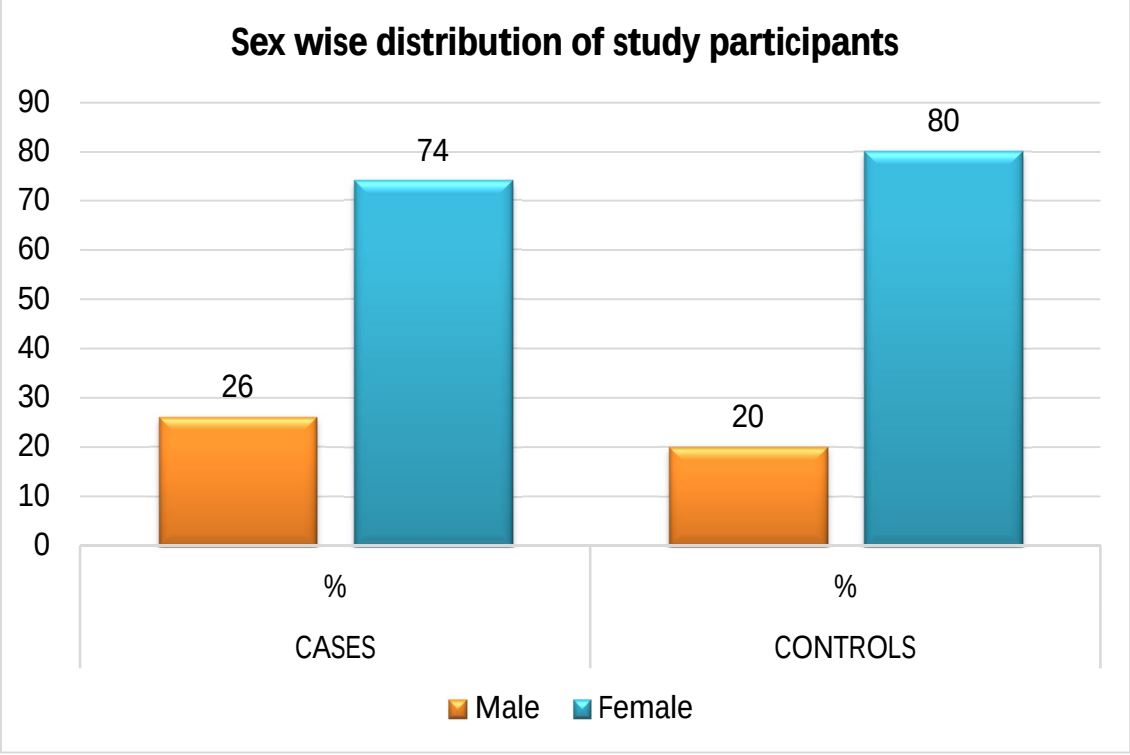


Table 2 : Distribution of study participants according to sex.

SEX	*Cases*		*controls*		*Percentage (%)*
	N	*%*	*N*	*%*	
Male	13	26	10	20	23
Female	37	74	40	80	77

Most of the study participants were females.

Figure 2 : Bar diagram showing the frequency of study participants according to sex.



74% of the cases were females and 80 % of the controls were females. Remaining 26% cases and 20% controls were males.

Table 3 : Age-sex wise distribution of cases and controls.

Age	*Male*		*Female*		*Percentage (%)*
	N	*%*	*N*	*%*	
< 20	0	0	5	6.4	5.0
21-30	3	13.6	16	20.5	19.0
31-40	10	45.5	32	41	42.0
41-50	7	31.8	16	20.5	23.0
51-60	2	9.1	9	11.5	11.0
Total	**22**	**22**	**78**	**78**	100.0

Figure 3 : Bar diagram showing the age and sex wise distribution of study population

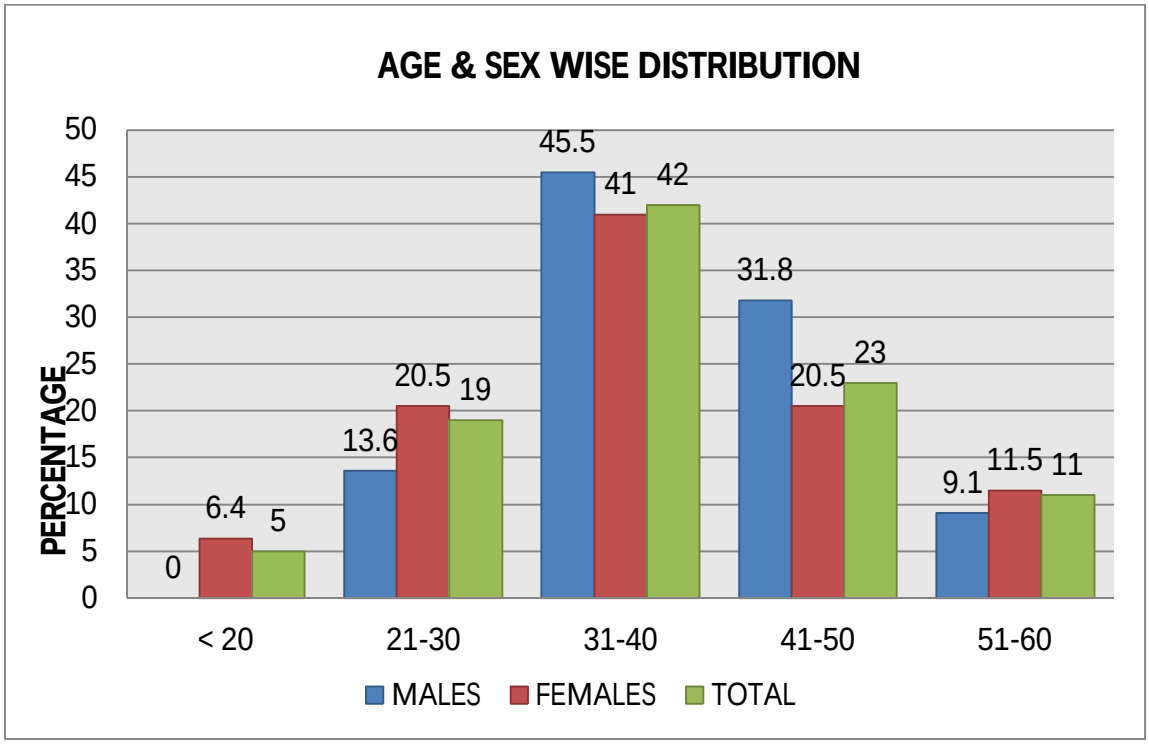
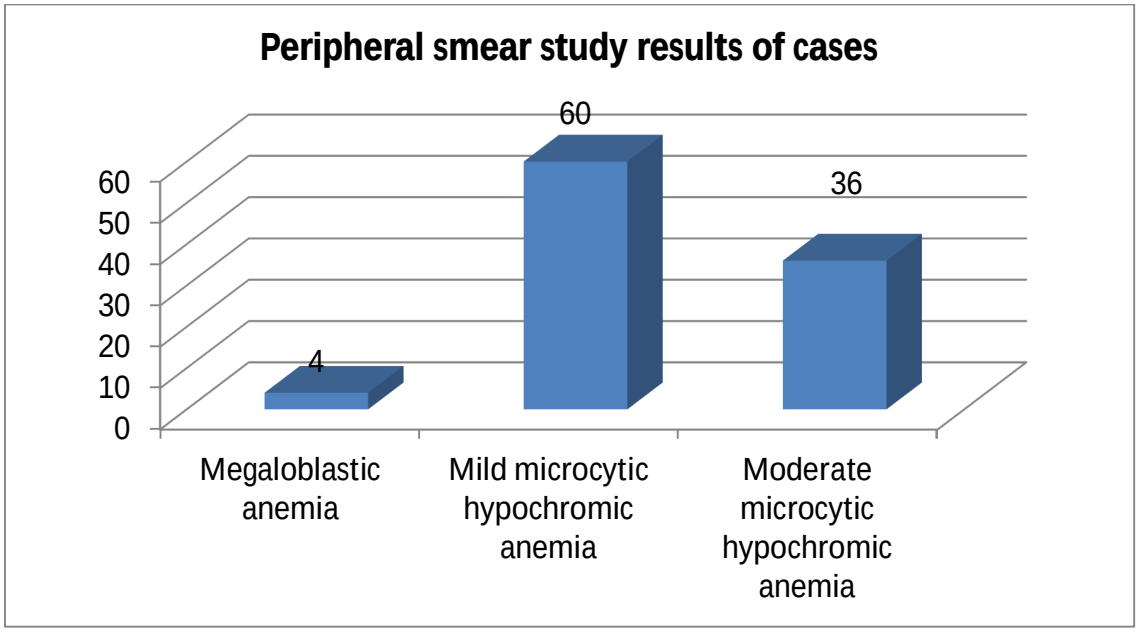


Table.4 Peripheral smear study results of cases

TYPE OF ANEMIA	*FREQUENCY*	*PERCENT*
Megaloblastic anemia	**2**	**4**
Mild microcytic hypochromic anemia	**30**	**60**
Moderate microcytic hypochromic anemia	**18**	**36**
Total	**50**	**100**

Normal peripheral smear study was observed among the control groups

Figure 4 : Bar diagram showing various types of anemia among study population.



Mild microcytic hypochromic anemia was the predominant type among the study population.

Table .6 Comparing variables used for diagnosing iron deficiency anemia between cases and controls.

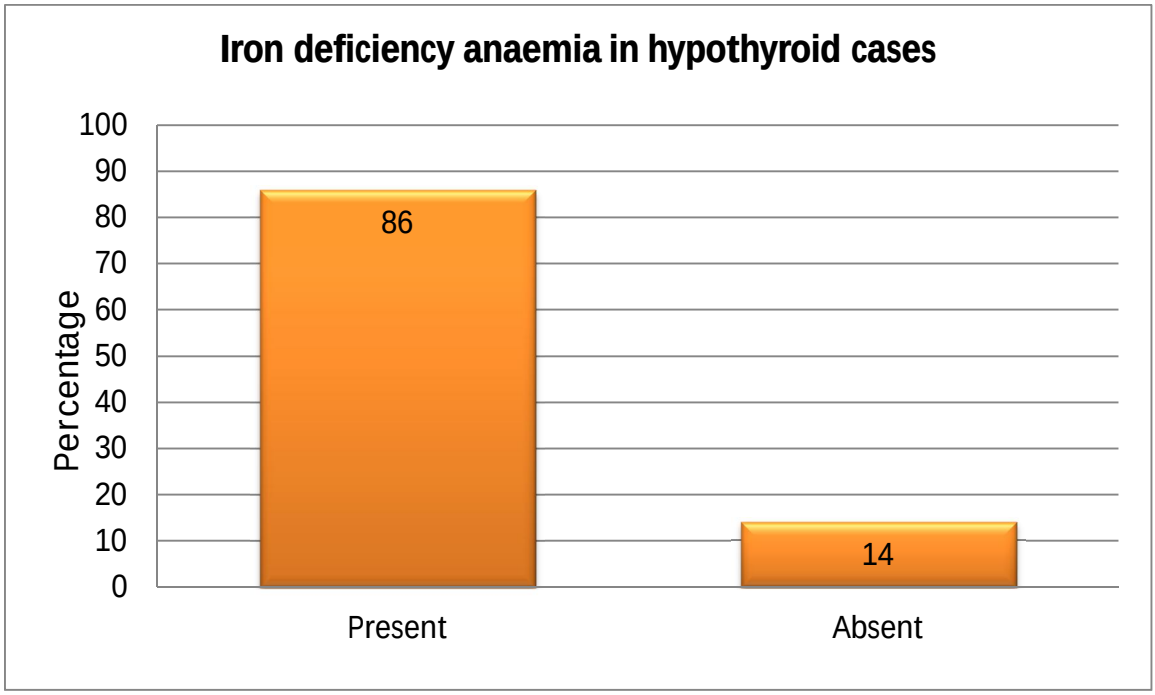
Variable	Study group	Mean	p value
Hb	Hypothyroid Cases	9.97	<0.001
	Euthyroid Controls	13.79	
Ferritin	Hypothyroid Cases	22.74	<0.001
	Euthyroid Controls	203.94	
sTfR	Hypothyroid Cases	3.57	<0.001
	Euthyroid Controls	1.40	
STfR/log ferritin	Hypothyroid Cases	3.32	<0.001
	Euthyroid Controls	0.73	

Table .7 Shows the comparison between iron deficiency anemia and hypothyroidism

Iron deficiency anaemia	Hypothyroidism	
	Present	
	N	%
Present	43	86
Absent	7	14
Total	50	100

Among the 50 hypothyroid cases , 43 showed iron deficiency anemia. Seven cases tested negative.

Figure 5 : Bar diagram showing percentage of iron deficiency anemia among hypothyroid patients



86% of hypothyroid cases had iron deficiency anemia.

Table.8 Comparison of Hemoglobin levels with thyroid hormone status

Variable		Hb (g/dL)	TSH (µIu/mL)	FT3 (pmol/L)	FT4 (ng/dL)
Hb (g/dL)	Pearson correlation	1	-0.522	0.655	0.541
	Significance 2 tailed		0.00	0.00	0.00
	N	100	100	100	100

From the above table, after comparing the Hb and thyroid hormone status among study population, a negative correlation exists between TSH and Hb and a positive correlation between Hb and fT3 ,fT4 levels.

Therefore when the Hb levels decreases, TSH value raises. On the otherhand, when the Hb levels increases , fT3 ,fT4 levels falls. Also the p value indicates that the correlation is statistically highly significant.

Fig 6. Comparison between TSH and Hb

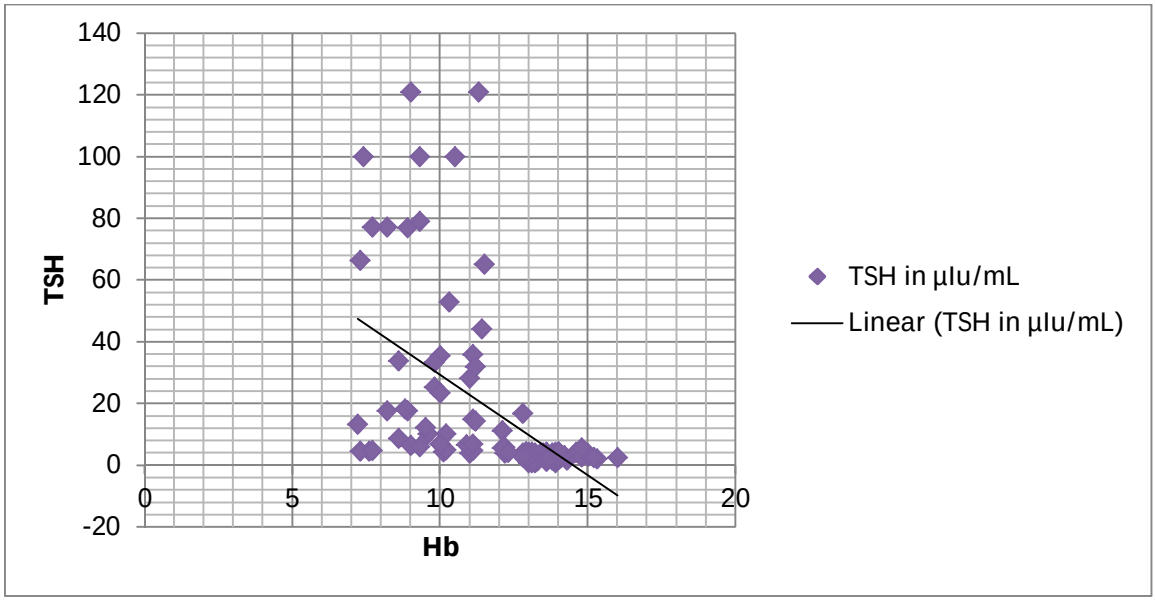


Fig 7. Scatter plot showing the comparison between fT3 and Hb

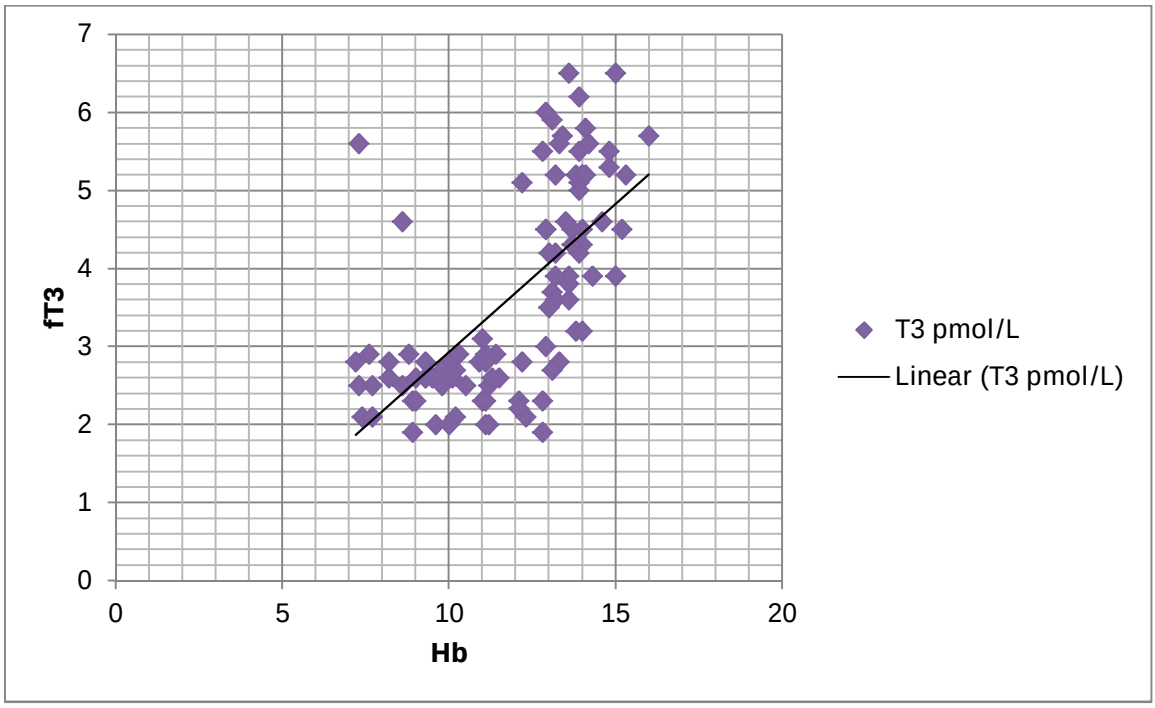


Fig 8. Scatter plot showing the comparison between fT4 and Hb

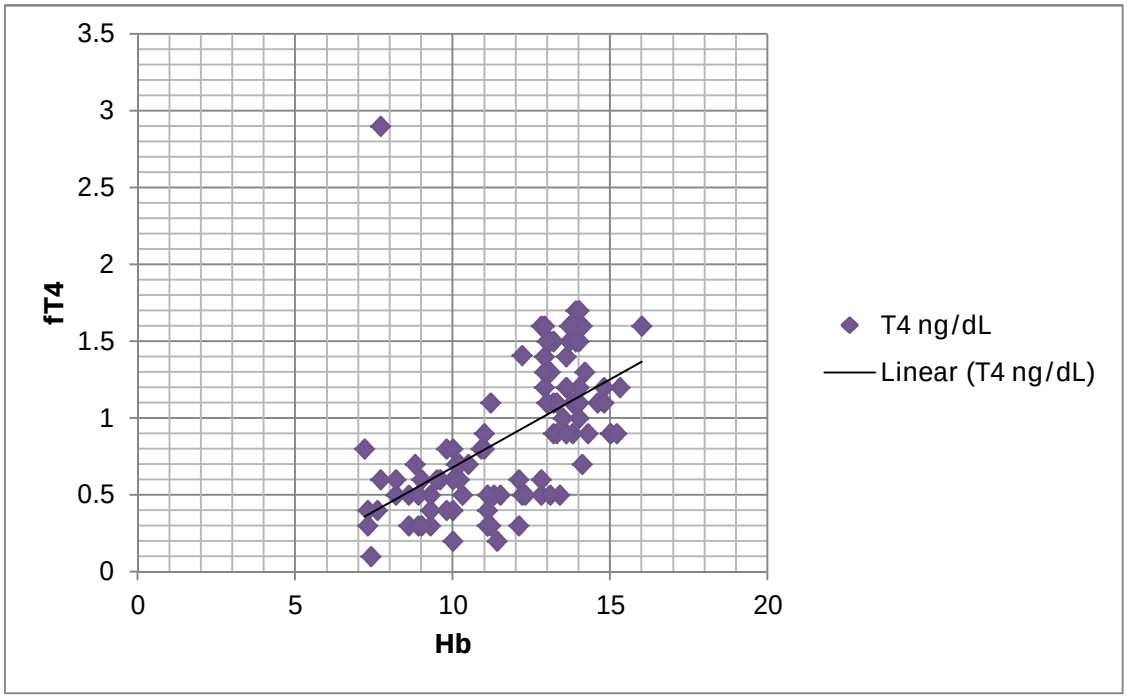


Table.9 Comparison of Ferritin levels with thyroid hormone status

Variable		Ferritin (µg/L)	TSH (µIu/mL)	FT3 (pmol/L)	FT4 (ng/dL)
	Pearson correlation	**1**	**-0.310**	**0.542**	**0.383**
Ferritin (µg/L)	Significance 2 tailed		0.00	0.00	0.00
	N	100	100	100	100

The above table implies that there exists a correlation among thyroid hormone status and ferritin levels. The p value which is 0.00 suggests a highly significant correlation.

Ferritin and TSH are negatively correlated. fT3 ,fT4 levels and ferritin are positively correlated.

Fig 9. Comparison between TSH and ferritin

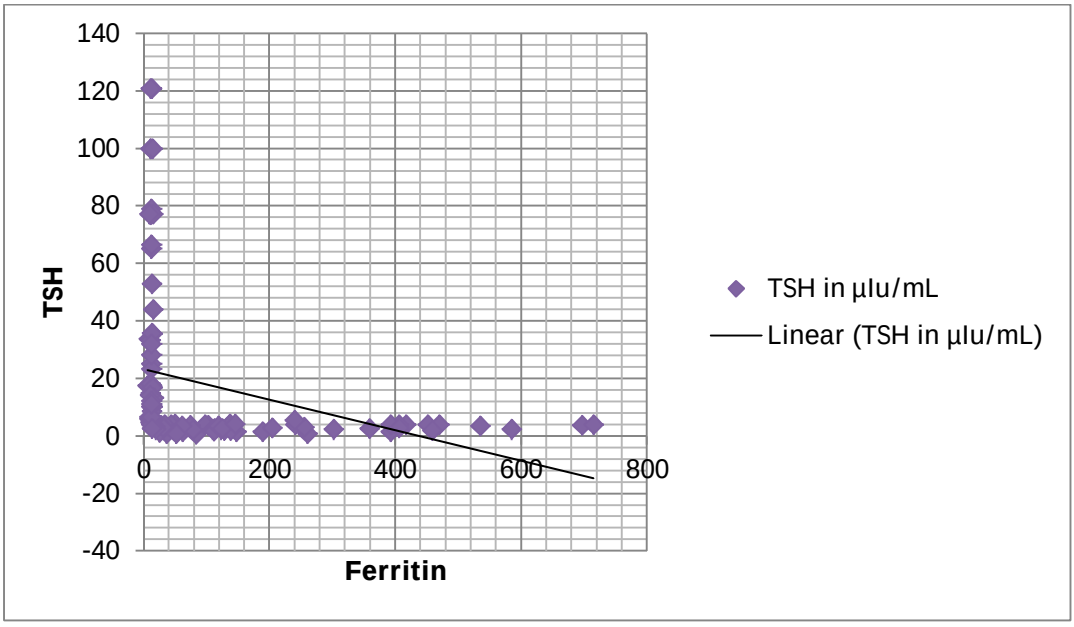


Fig 10. Scatter plot showing the comparison between fT3 and ferritin

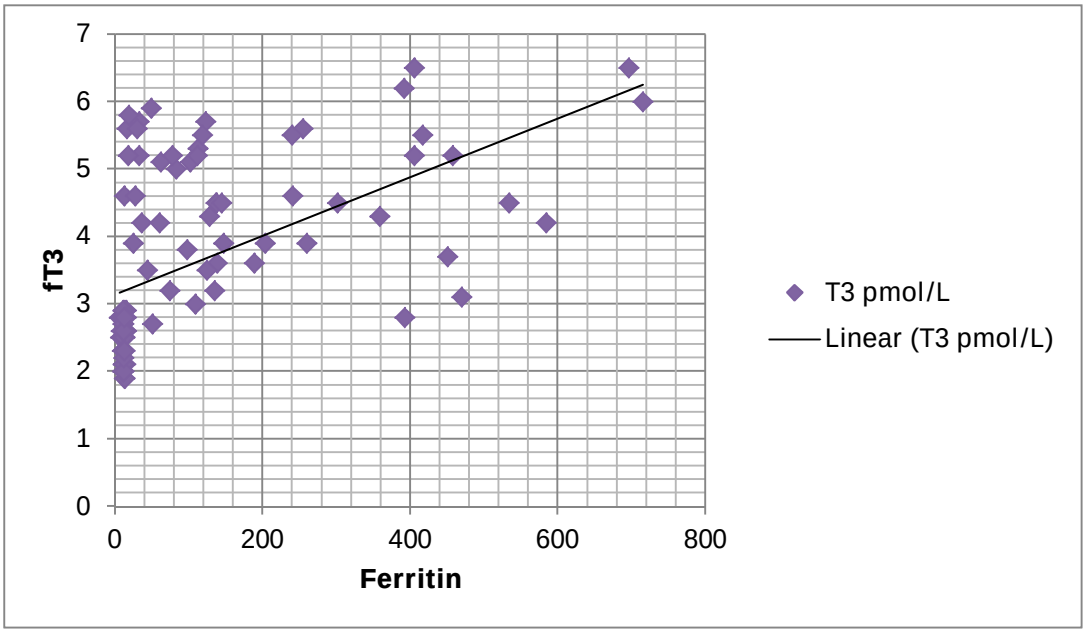


Fig 11. Scatter plot showing the comparison between fT4 and ferritin

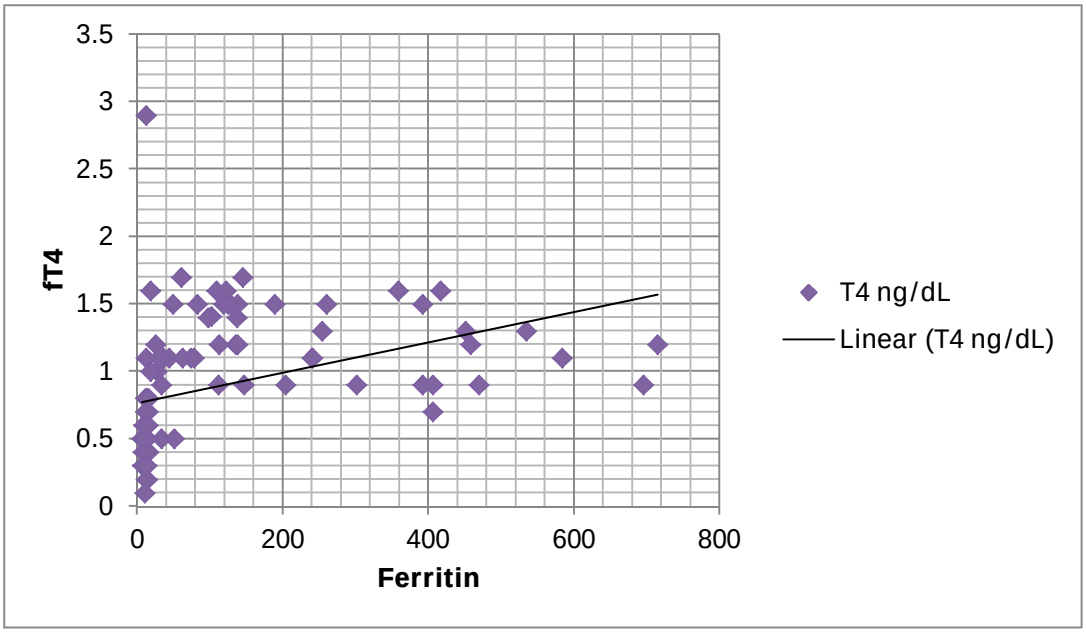


Table.10 Comparison of sTfR levels with thyroid hormone status

Variable		sTfR (mg/L)	TSH (µIu/mL)	FT3 (pmol/L)	FT4 (ng/dL)
	Pearson correlation	**1**	**0.444**	**-0.604**	**-0.597**
sTfR (mg/L)	Significance 2 tailed		0.00	0.00	0.00
	N	100	100	100	100

The above table implies that there exists a correlation among thyroid hormone status and sTfR levels. The p value which is 0.00 suggests a highly significant correlation.

sTfR and TSH are positively correlated

fT3 ,fT4 levels and sTfR are negatively correlated

Fig 12. Comparison between TSH and sTfR

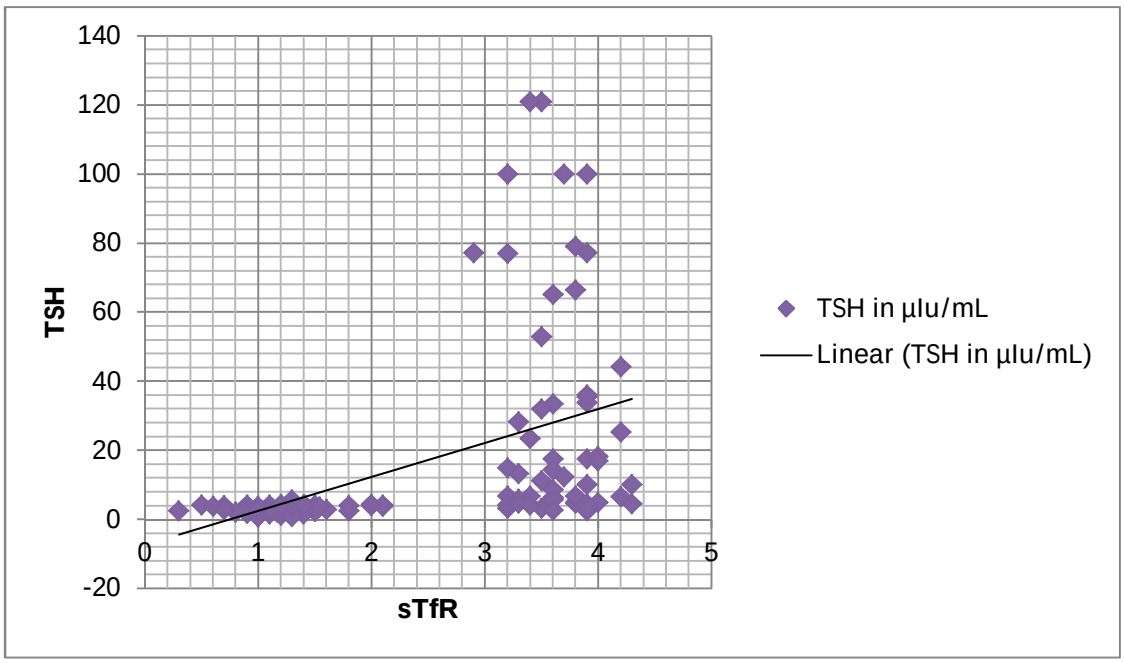


Fig 13. Scatter plot showing the comparison between fT3 and sTfR

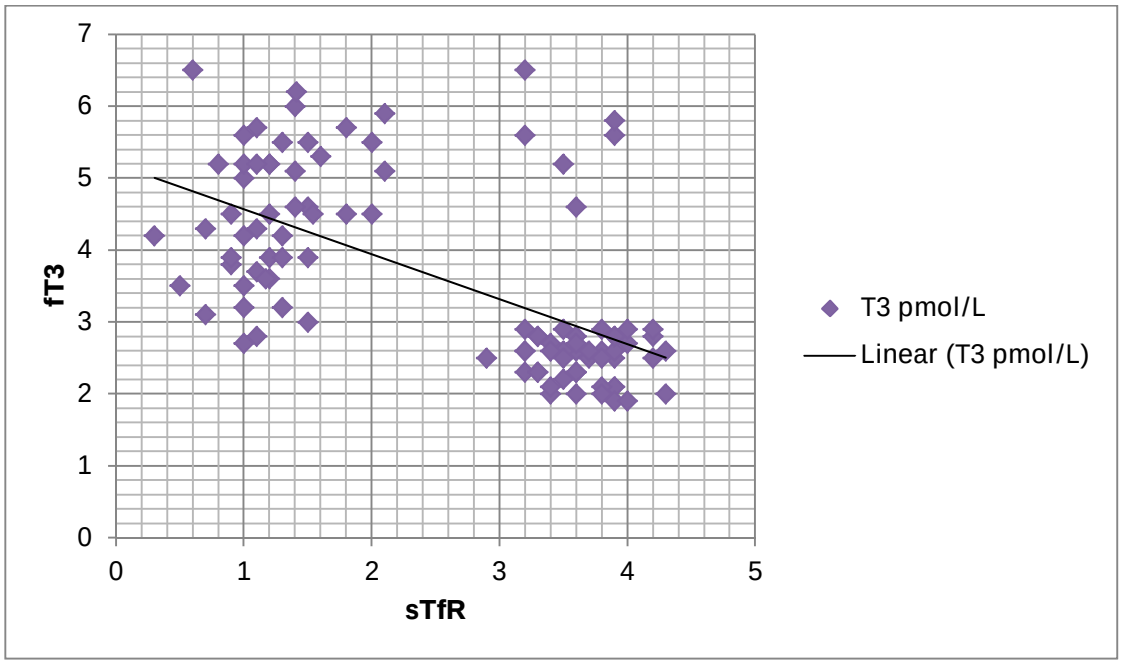


Fig 14. Scatter plot showing the comparison between fT4 and sTfR

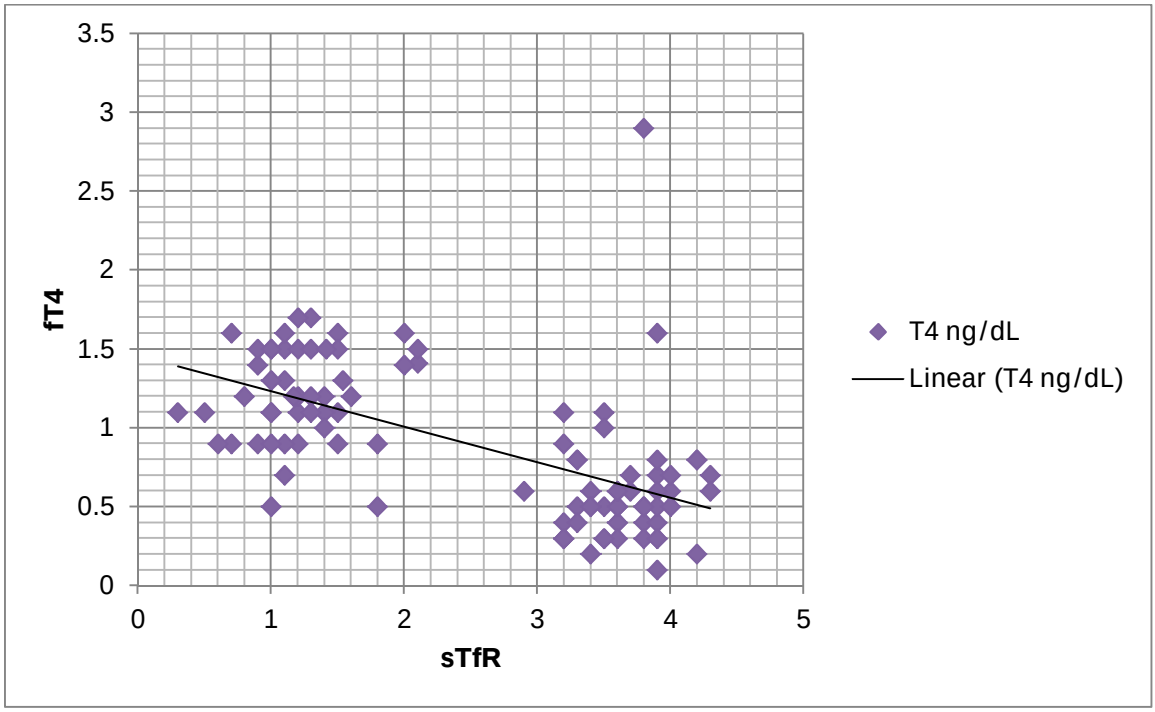


Table.11 Comparison of sTfR /log ferritin levels with thyroid hormone status

Variable		sTfR /log ferritin	TSH (µIu/mL)	FT3 (pmol/L)	FT4 (ng/dL)
	Pearson correlation	**1**	**0.475**	**-0.692**	**-0.651**
sTfR /log ferritin	Significance 2 tailed		0.00	0.00	0.00
	N	100	100	100	100

The above table implies that there exists a correlation among thyroid hormone status and sTfR/log ferritin levels. The p value which is 0.00 suggests a highly significant correlation.

sTfR/log ferritin and TSH are positively correlated

fT3 ,fT4 levels and sTfR/log ferritin are negatively correlated

Fig 15. Comparison between TSH and sTfR/log ferritin

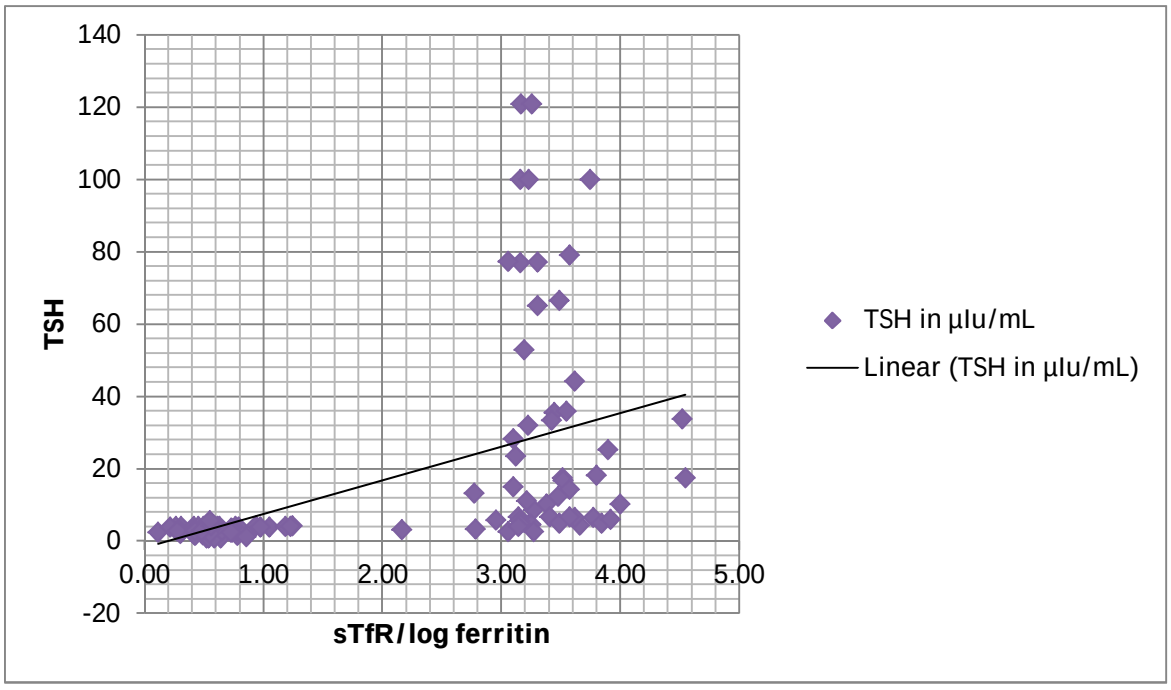


Fig 16. Comparison between fT3 and sTfR/log ferritin

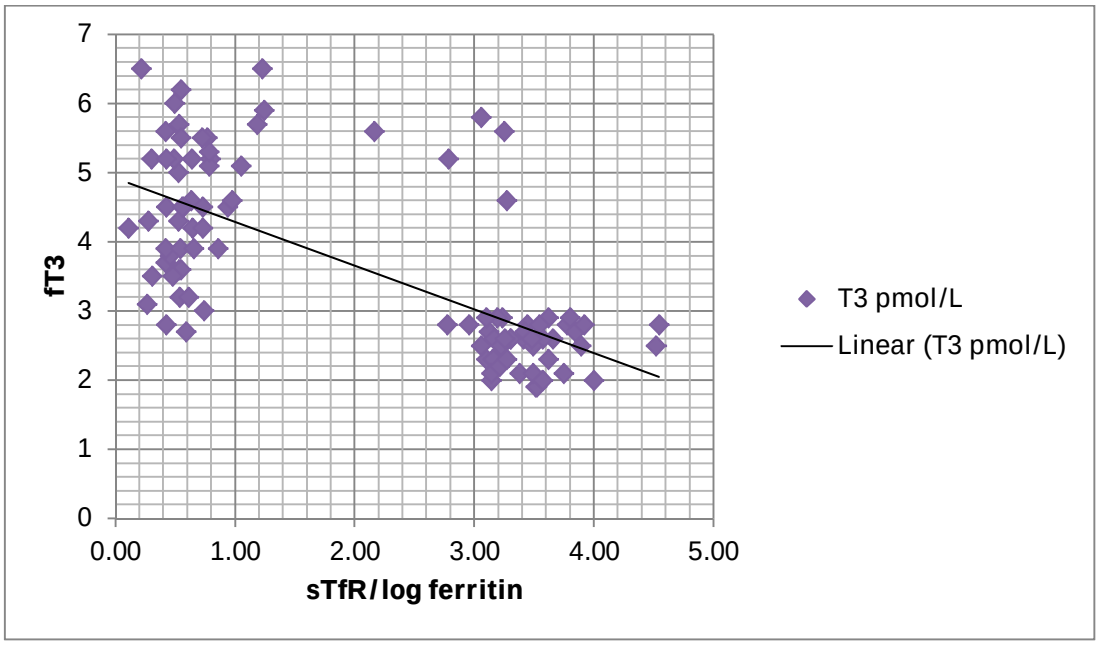


Fig 17. Comparison between fT4 and sTfR/log ferritin

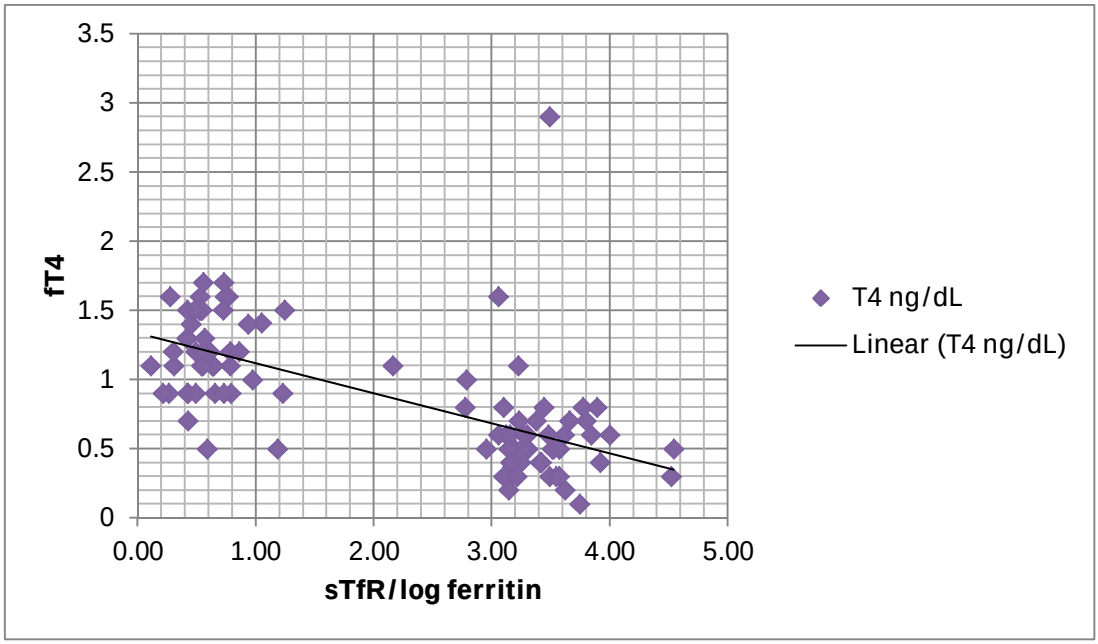
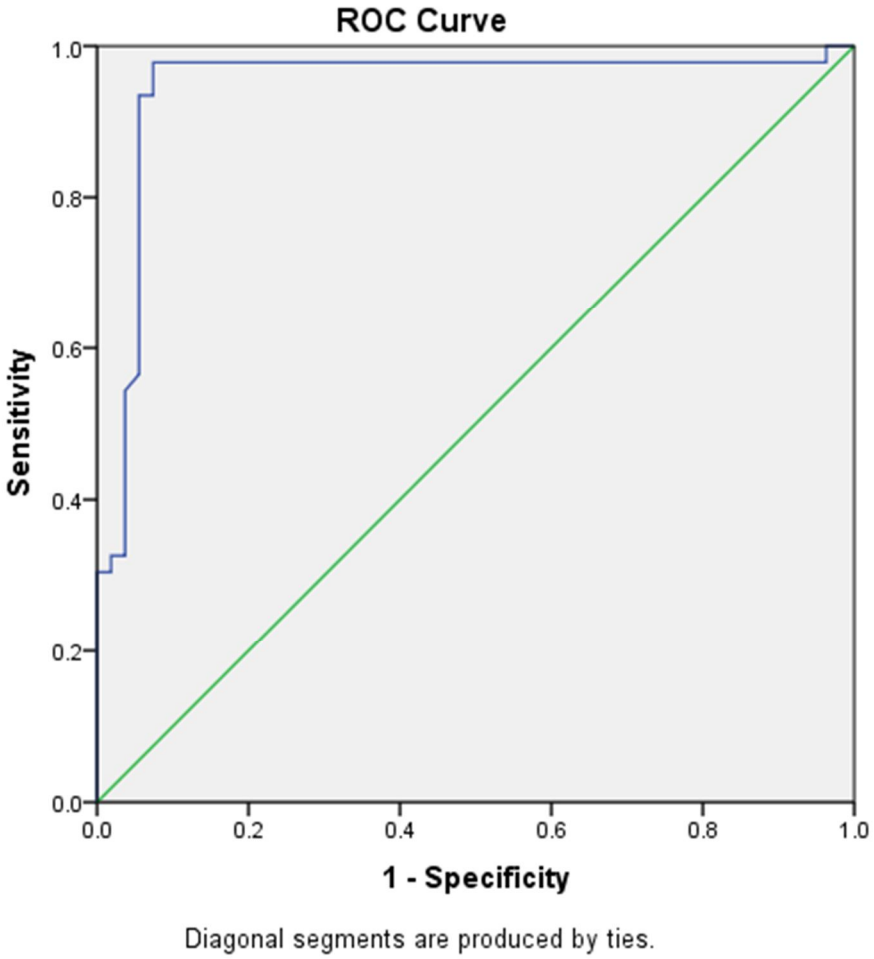


CHART SHOWING THE COMPARISON BETWEEN DIFFERENT ANALYTES								
		HbinCBC	FERRITIN	sTfRinmgL	sTfRlogferritin	TSHinµIumL	T3pmolL	T4ngdL
HbinCBC	Pearson Correlation	1	.496**	-.760**	-.811**	-.522**	.655**	.541**
	Sig. (2-tailed)		.000	.000	.000	.000	.000	.000
	N	99	99	99	99	99	99	99
FERRITIN	Pearson Correlation	.496**	1	-.617**	-.660**	-.310**	.542**	.383**
	Sig. (2-tailed)	.000		.000	.000	.002	.000	.000
	N	99	100	100	100	100	100	100
sTfRinmgL	Pearson Correlation	-.760**	-.617**	1	.970**	.444**	-.604**	-.597**
	Sig. (2-tailed)	.000	.000		.000	.000	.000	.000
	N	99	100	100	100	100	100	100
sTfRlogferritin	Pearson Correlation	-.811**	-.660**	.970**	1	.475**	-.692**	-.651**
	Sig. (2-tailed)	.000	.000	.000		.000	.000	.000
	N	99	100	100	100	100	100	100
TSHinµIumL	Pearson Correlation	-.522**	-.310**	.444**	.475**	1	-.421**	-.461**
	Sig. (2-tailed)	.000	.002	.000	.000		.000	.000
	N	99	100	100	100	100	100	100
T3pmolL	Pearson Correlation	.655**	.542**	-.604**	-.692**	-.421**	1	.527**
	Sig. (2-tailed)	.000	.000	.000	.000	.000		.000
	N	99	100	100	100	100	100	100
T4ngdL	Pearson Correlation	.541**	.383**	-.597**	-.651**	-.461**	.527**	1
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	
	N	99	100	100	100	100	100	100

**. Correlation is significant at the 0.01 level (2-tailed).

Significant correlations can be seen among the thyroid hormones and Hb, ferritin, sTfR , sTfR/log ferritin levels.

ROC curve for sTfR/log ferritin to diagnose IDA in hypothyroid cases



Area
.946
The test result variable(s): sTfRlogferritin has at least one tie between the positive actual state group and the negative actual state group.

Discussion

DISCUSSION

Thyroid diseases are common worldwide. Thyroid hormones are very important to regulate the metabolism of human body. Hypothyroidism is more prevalent among the female population[66] [68]. There exists a labrynthine connection between thyroid metabolism and iron metabolism. Though there are many evidences for this relationship among rats only very few literatures are available to comment this status in human beings

This cross sectional case control study was conducted in the view to appraise iron levels in hypothyroidism patients and euthyroid control group. In this study, the study population is divided into 2 groups

Group A: CASES: Recently diagnosed hypothyroid patients

Group 2: CONTROLS : Healthy volunteers

AGE

Iron deficiency is more common among adolescent girls(5) but hypothyroidism is commonly seen among the middle age groups. So we have decided to go for the population of age group between 15 to 60 years. In the geriatric population there is a high risk for other comorbid conditions which may affect the iron levels. So we have excluded the population more than 60 years. Since we need to establish a correlation between hypothyroid and iron deficient

patients, hypothyroidism being common in middle age people, we have excluded the pediatric population .

Mean value of the age for the cases were 37.63 and the controls were 36.48. p value was 0.28(>0.05) which means the difference is not statistically significant. The overall mean age of the study participants were 37.07±9.90 years. Most of the study participants were in the age group of 31-40 years (42%)

Diagnosis of iron deficiency anemia is usually done by an amalgam of clinical symptoms, signs and laboratory investigations. The conventional laboratory parameters include iron levels, ferritin and TIBC levels. But these markers are found to be less sensitive and less specific for diagnosing iron deficiency anemia because these can be affected by any chronic inflammatory diseases.[(69),(28)]

So we used newer markers such as sTfR, sTfR/log ferritin along with ferritin to measure iron status which , according to Lee et al (59) and Infusino et al[(28)] had better sensitivities and specificities.

SEX

According to Dahiya et al[(66)], proclivity of hypothyroidism is 60 % in females. Because women have estrogen which has an anti- thyroid effect. Also iron deficiency anemia is common among females. In our study 74% of the females were found to be hypothyroid.

PERIPHERAL SMEAR REPORT

Peripheral smear study shows that 30 cases tested positive for mild microcytic anemia , 18 cases tested positive for moderate microcytic anemia and 2 showed megaloblastic anemia among the total of 50 cases of the study group.

Hb

Majority of the patients in the study group had low Hb which was statistically significant when compared with controls ($p<0.05$) and is similar to the study by Chua et al[32].

Ferritin

The ferritin value was lower among the cases shows statistically significant difference among cases and controls ($p<0.05$)

sTfR

It is the best marker to estimate iron levels because it is not affected by inflammation compared to other parameters[32]. Also it is raised in almost all the cases of iron deficiency anemia . In this study the sTfR value shows statistically significant difference among cases and controls ($p<0.05$) .

sTfR/log ferritin

The sTfR/log ferritin value shows statistically significant difference among cases and controls ($p<0.05$). It is also elevated in a vast majority of IDA cases[34].

It is only during the past decade, the significance of sTfR and sTfR / log ferritin levels over iron and TIBC in the diagnoses of IDA has been observed. sTfR is an early marker for diagnosing iron deficiency being unaffected by inflammation while sTfR-log ferritin covers the entire spectrum of body iron status, from normality to iron deficient tissue[70] ,[71], [72].

CORRELATION

In our study we compared thyroid hormones fT3, fT4 and TSH which are used in diagnosing hypothyroidism with the parameters for diagnosing iron deficiency anemia such as Hb, ferritin, sTfR and sTfR/log ferritin levels using Pearson's coefficient formula. Correlation was done in an aim to establish a relationship between hypothyroidism and iron deficiency anemia.

Results of our correlation study were :

- A negative correlation existed between TSH and Hb and a positive correlation between Hb and fT3 ,fT4 levels which was statistically highly significant. ($p<0.01$)
- Ferritin and TSH are negatively correlated and
  fT3 ,fT4 levels and ferritin are positively correlated
  and the correlation was statistically highly significant ($p<0.01$).

The results of our study are similar to the study by Dahiya et al[67]

- When comparing sTfR and TSH we found a positive correlation while fT3, fT4 levels negatively correlated with sTfR with a high statistical significance ($p < 0.01$).

ROC curve for sTfR/log ferritin to diagnose IDA in hypothyroid cases

Area under the curve was 0.946. Also the cut off point to diagnose IDA from the curve was 3.07 which had 94% sensitivity and a good specificity. So this proves that sTfR /log ferritin is an essential marker to diagnose IDA in addition to sTfR and ferritin levels.

So using the above parameters to diagnose hypothyroidism and iron deficiency anemia, our study showed that among 50 hypothyroid patients, 43 tested positive for iron deficiency anemia, i.e.,86% of hypothyroid cases had iron deficiency anemia.

This shows the existence of a mutual relationship between IDA and hypothyroidism.

Conclusion

CONCLUSION

The present study was conducted with an aim to study the iron status among hypothyroid patients and correlate the ferritin & soluble transferrin receptor levels with thyroid profile in hypothyroid patients . The study population consisted of 50 cases of proven hypothyroid patients (37 females and 13 males) with an average age of 37. The control group consisted of 50 healthy volunteers (40 females and 10 males) and were age matched with that of controls.

The study population was categorized into five groups based on age as <20, 21- 30, 31- 40, 41-50 and 51- 60.

From this study we conclude that:

1. Hypothyroidism is more common among the women aged 31- 40 years.
2. Ferritin levels were found to be decreased in hypothyroid cases as compared to the control group
3. sTfR and sTfR/log ferritin levels were found to be increased in hypothyroid cases as compared to the control group and also found to be an excellent marker for IDA.
4. Almost all of the cases (96%) showed microcytic hypochromic anemia in their peripheral smear study which is characteristic of IDA.
5. Ferritin decreases with increase of TSH and is elevated with decline of fT3 and fT4,i.e., it is negatively correlated with TSH, fT3 and fT4.

6. sTfR and sTfR/log ferritin levels increases with increased TSH and decreases with the fall in fT3 and fT4 levels showing a positive correlation.
7. sTfR, sTfR/log ferritin levels were found to be a better marker to diagnose IDA.
8. Iron deficiency anemia is a characteristic feature seen in hypothyroidism and from our study we have observed the existence of a mutual relationship between hypothyroidism and IDA. Hence we conclude that hypothyroidism may be a cause and effect of iron deficiency anemia and vice versa.

Limitation of the Study

LIMITATIONS OF THE STUDY

- Reference ranges are not established for sTfR, sTfR/log ferritin levels among the Indian population
- Most of the study participants were females. So it was difficult to establish the significance of sTfR, sTfR/log ferritin levels in diagnosing IDA among male population
- Majority of the Indian women have nutritional iron deficiency. It is highly difficult to differentiate nutritional iron deficiency among them.
- In the absence of nutritional iron deficiency, the exact mechanism of IDA in hypothyroidism is not understood. Further studies on the enzyme thyroid peroxidase may provide more information.

Future Prospects

FUTURE PROSPECTS

- sTfR, sTfR/log ferritin can be used in the test panel to estimate serum iron levels
- sTfR,sTfR/log ferritin can be used as an marker to diagnose iron deficiency and to differentiate the anemia in patients with chronic inflammatory conditions.
- Studies to establish reference ranges for sTfR, sTfR/log ferritin among Indian population
- All hypothyroid patients shall be screened for iron deficiency anemia
- All iron deficiency patients shall be tested for hypothyroidism
- Studies on the enzyme thyroid peroxidase to understand the cause for the mutual relationship between iron deficiency anemia and hypothyroidism may provide more information.
- Randomized control trials can be done on hypothyroid patients along with measuring thyroid hormones before and after iron supplementation.

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Annexures

INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI 600 003

EC Reg.No.ECR/270/Inst./TN/2013
Telephone No.044 25305301
Fax: 011 25363970

CERTIFICATE OF APPROVAL

To
Dr.K.S.Gokilaveni
I Year PG in MD Bio-Chemistry
Institute of Bio-Chemistry
Madras Medical College
Chennai 600 003

Dear Dr.K.S.Gokilaveni,

The Institutional Ethics Committee has considered your request and approved your study titled "**A STUDY ON IRON STATUS & THYROID FUNCTION: ? MUTUAL RELATIONSHIP IN HYPOTHYRODISM " - NO.13032017(I)**

The following members of Ethics Committee were present in the meeting hold on **02.03.2017** conducted at Madras Medical College, Chennai 3

1.Dr.C.Rajendran, MD.,	:Chairperson
2.Dr. K.Narayanasamy,MD,DM.,Dean(FAC), MMC,Ch-3	:Deputy Chairperson
3.Prof.Sudha Seshayyan,MD., Vice Principal,MMC,Ch-3	: Member Secretary
4.Prof.S.Suresh, MS, Prof. of Surgery,MMC,Ch-3	: Member
5.Prof.Baby Vasumathi,MD.,Director, Inst. of O & G	: Member
6.Prof.K.Ramadevi,MD.,Director,Inst.of Bio-Che,MMC,Ch-3	: Member
7.Prof.R.Padmavathy, MD, Director,Inst.of Pathology,MMC,Ch-3	: Member
8.Tmt.J.Rajalakshmi, JAO,MMC, Ch-3	: Lay Person
9.Thiru S.Govindasamy, BA.,BL,High Court,Chennai	: Lawyer
10.Tmt.Arnold Saulina, MA.,MSW.,	:Social Scientist

We approve the proposal to be conducted in its presented form.

The Institutional Ethics Committee expects to be informed about the progress of the study and SAE occurring in the course of the study, any changes in the protocol and patients information/informed consent and asks to be provided a copy of the final report.

Member Secretary - Ethics Committee

MEMBER SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE
CHENNAI-600 003

URKUND

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PLAGIARISM CERTIFICATE

This is to certify that this dissertation work titled **"A STUDY ON IRON STATUS & THYROID FUNCTION : ? MUTUAL RELATIONSHIP IN HYPOTHYROIDISM"** of the candidate **Dr. K.S.GOKILAVENI** with registration Number **201623002** for the award of **M.D** in the branch of **BIOCHEMISTRY**. I personally verified the urkund.com website for the purpose of plagiarism Check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **3 percentage** of plagiarism in the dissertation.

Guide & Supervisor sign with Seal.

PROFORMA

Name :

Age/sex:

IP/OP No:

Ward:

History:

Examination :

Investigations:

1.Fasting Thyroid Profile

TSH :

T3 :

T4 :

2.Complete Blood count :

3.Hb :

4.Serum Ferritin :

5.Serum Soluble transferrin receptor protein :

6.Peripheral smear study :

7. Soluble transferrin receptor:log Ferritin :

INFORMATION SHEET

- Your blood sample has been accepted.
- We are conducting a study on patients with diagnosed thyroid disorders at Rajiv Gandhi Government General Hospital, Chennai and for that your blood sample may be valuable to us.
- The purpose of this study is to correlate the level of serum ferritin and serum soluble transferrin receptor protein levels in patients with diagnosed thyroid disorders with the help of certain special tests.
- We are selecting certain cases and if your blood sample is found eligible, we may be using your blood sample to perform extra tests and special studies which in any way do not affect your final report or management.
- The privacy of the patients in the research will be maintained throughout the study. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.
- Taking part in this study is voluntary. You are free to decide whether to participate in this study or to withdraw at any time; your decision will not result in any loss of benefits to which you are otherwise entitled.
- The results of the special study may be intimated to you at the end of the study period or during the study if anything is found abnormal which may aid in the management or treatment.

Signature of investigator Signature of participant

Date:

PATIENT CONSENT FORM

Title of the study: **A STUDY ON IRON STATUS & THYROID FUNCTION : ?MUTUAL RELATIONSHIP IN HYPOTHYROIDISM"**

Name : Date :

Age : OP No:

Sex : Project Patient No :

The details of the study have been provided to me in writing and explained to me in my own language.

I confirm that I have understood the above study and had the opportunity to ask questions.

I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected.

I agree to use my personal clinical history& investigation details for the purpose of the study.

I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s).

I have been given an information sheet giving details of the study.

Having understood _____s/o_____ give my consent to participate in the study conducted by DR.K.S.GOKILAVENI, Post graduate, Institute of Biochemistry, Madras Medical College, Chennai.

Signature of the investigator: Signature of the participant

Place: Thumb impression.

Date:

ஆராய்ச்சி தகவல் தாள்.

தங்களது இரத்தம் இங்குபெற்றுக்கொள்ளப்பட்டது.

சென்னை அரசுபொது மருத்துவமனையில் "**தைராய்டு சுரப்பியில் குறைபாடுள்ள நோயாளிகளில் இரும்புச் சத்து பற்றிய ஓர் ஆய்வு**" என்ற தலைப்பில் ஆராய்ச்சி நடைபெற்று வருகின்றது.

நீங்களும் இந்த ஆராய்ச்சியில் பங்கேற்க நாங்கள் விரும்பிகிறோம் .

இந்த ஆராய்ச்சியில் உங்களுடைய இரத்தம் எடுத்து சிறப்புப்பரி சோதனைக்கு உட்படுத்தி அதன் தகவல்களை ஆராய்வோம்.

அதனால் தங்களது நோயின் ஆய்வறிக்கையோ அல்லது சிகிச்சையோ பாதிப்புக்கு உள்ளாகாது என்பதையும் தெரிவித்துக்கொள்கிறோம்.

முடிவுகளை அல்லது கருத்துக்களை வெளியிடும் போதோ அல்லது ஆராய்ச்சியின் போதோ தங்களது பெயரையோ அல்லது அடையாளங்களையோ வெளியிடமாட்டோம் என்பதையும் தெரிவித்துக்கொள்கிறோம்

இந்த ஆராய்ச்சியில் பங்கேற்பது தங்களுடைய விருப்பத்தின் பேரில் தான் இருக்கிறது.மேலும் நீங்கள் எந்நேரமும் இந்த ஆராய்ச்சியிலிருந்து பின்வாங்கலாம் என்பதையும் தெரிவித்துகொள்கிறோம்.

இந்த சிறப்பு பரிசோதனைகளின் முடிவுகளை ஆராய்ச்சியின் போது அல்லது ஆராய்ச்சியின் முடிவின் போது தங்களுக்கு அறிவிப்போம் என்பதையும் தெரிவித்துக்கொள்கிறோம்.

ஆராய்ச்சியாளர் கையொப்பம் பங்கேற்பாளர் கையொப்பம்

தேதி:

நோயாளியின்ஒப்புதல்படிவம்

ஆராய்ச்சி தலைப்பு: **தைராய்டு சுரப்பியில் குறைபாடுள்ள நோயாளிகளில் இரும்புச் சத்து பற்றிய ஓர் ஆய்வு.**

பெயர் : தேதி :

வயது : புறநோயாளிஎண்:

பால் : ஆராய்ச்சி சேர்க்கை எண் :

இந்த ஆராய்ச்சியின் விவரங்களும் அதன் நோக்கங்களும் முழுமையாக எனக்கு தெளிவாக விளக்கப்பட்டது.

எனக்கு விளக்கப்பட்ட விஷயங்களை புரிந்து கொண்டு நான் எனது சம்மதத்தைத் தெரிவிக்கிறேன்.

இந்த ஆராய்ச்சியில் பிறரின் நிர்பந்தமின்றி என் சொந்த விருப்பத்தின் பேரில் தான் பங்கு பெறுகிறேன் மற்றும் நான் இந்த ஆராய்ச்சியிலிருந்து எந்நேரமும் பின்வாங்கலாம் என்பதையும், அதனால் எந்த பாதிப்பும் ஏற்படாது என்பதையும் புரிந்து கொண்டேன்நான் **"தைராய்டு சுரப்பியில் குறைபாடுள்ள நோயாளிகளில் இரும்புச் சத்து பற்றிய ஓர் ஆய்வு"** என்ற தலைப்பில் மேற்கொள்ளப்படும் இந்த ஆராய்ச்சியின் விபரங்களைக் கொண்ட தகவல் தாளைப் பெற்றுக் கொண்டேன்.

இதன் மூலம் எந்த பின்விளைவும் வராது என மருத்துவர் மூலம் தெரிந்து கொண்டு என்னுடைய சுயநினைவுடன் மற்றும் முழு சுதந்திரத்துடன் இந்த மருத்துவ ஆராய்ச்சியில் என்னை சேர்த்துக்கொள்ள சம்மதிக்கிறேன்

தேதி கையொப்பம்